ACS SYMPOSIUM SERIES [illegible]

Natural Products for Pest Management

EDITED BY

Agnes M. Rimando and Stephen O. Duke

Natural Products for Pest Management

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.fw001

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.fw001

ACS SYMPOSIUM SERIES **927**

Natural Products for Pest Management

Agnes M. Rimando, Editor
Agricultural Research Service, U.S. Department of Agriculture

Stephen O. Duke, Editor
Agricultural Research Service, U.S. Department of Agriculture

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.fw001

Sponsored by the
ACS Division of Agricultural and
Food Chemistry, Inc.

American Chemical Society, Washington, DC

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.fw001

Library of Congress Cataloging-in-Publication Data

Natural products for pest management / Agnes M. Rimando, editor, Stephen O. Duke, sponsored by the ACS Division of Agricultural and Food Chemistry, Inc.

p. cm.—(ACS symposium series ; 927)

Includes bibliographical references and indexes.

ISBN 13: 978–0–8412–3933–3 (alk. paper)

1. Natural pesticides—Congresses.

I. Rimando, Agnes M., 1957- II. Duke, Stephen O., 1944- III. American Chemical Society. Division of Agricultural and Food Chemistry, Inc. IV. Series.

SB951.145.N37N385 2006
632′.95—dc22 2005044496

The paper used in this publication meets the minimum requirements of American National Standard for Information Sciences—Permanence of Paper for Printed Library Materials, ANSI Z39.48–1984.

Copyright © 2006 American Chemical Society

Distributed by Oxford University Press

ISBN 10: 0–8412–3933–9

All Rights Reserved. Reprographic copying beyond that permitted by Sections 107 or 108 of the U.S. Copyright Act is allowed for internal use only, provided that a per-chapter fee of $33.00 plus $0.75 per page is paid to the Copyright Clearance Center, Inc., 222 Rosewood Drive, Danvers, MA 01923, USA. Republication or reproduction for sale of pages in this book is permitted only under license from ACS. Direct these and other permission requests to ACS Copyright Office, Publications Division, 1155 16th Street, N.W., Washington, DC 20036.

The citation of trade names and/or names of manufacturers in this publication is not to be construed as an endorsement or as approval by ACS of the commercial products or services referenced herein; nor should the mere reference herein to any drawing, specification, chemical process, or other data be regarded as a license or as a conveyance of any right or permission to the holder, reader, or any other person or corporation, to manufacture, reproduce, use, or sell any patented invention or copyrighted work that may in any way be related thereto. Registered names, trademarks, etc., used in this publication, even without specific indication thereof, are not to be considered unprotected by law.

PRINTED IN THE UNITED STATES OF AMERICA

Foreword

The ACS Symposium Series was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of the series is to publish timely, comprehensive books developed from ACS sponsored symposia based on current scientific research. Occasionally, books are developed from symposia sponsored by other organizations when the topic is of keen interest to the chemistry audience.

Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previously published papers are not accepted.

ACS Books Department

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.fw001

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.pr001

Preface

Citronella, *Derris* spp., and *Eucalyptus* spp. are examples of natural products that were used for pest control for centuries, but since the middle of the last century synthetic pesticides have largely replaced these traditional materials. There is now a resurgence of interest in natural products for pest management because of concerns over potential undesirable health and environmental effects of synthetic pesticide use, evolving resistance to synthetic pesticides, and the need for pesticides with novel modes of action. Advances in analytical instrumentation and assay methodologies have facilitated the discovery of new chemical entities from natural sources. Furthermore, molecular biology and transgene technology have provided the means of efficiently producing these compounds by biosynthesis, either by fermentation or directly in a crop for its protection. This is a growing area of research, and it is an opportune time to assemble a volume on the research of leaders in the field of pest management with natural products.

This volume was developed from a symposium which took place at the 226th ACS National Meeting on March 28–April 1, 2004 in Anaheim, California. This book covers a wide range of topics, including herbicides, insecticides, fungicides, algaecides, and molluscicides. The chapters provide discussions on newly discovered compounds, modes of action, genomics-based approaches in the discovery and characterization of natural product pest management agents, the use of transgenes to impart or enhance production of natural pest protectants in crops, new chemical methods to enhance pesticidal activity, and regulatory aspects governing natural products registration and commercialization. We hope that this collection of chapters by an international group of experts will be a valuable resource tool for those working to find alternatives to synthetic pesticides.

We appreciate the contribution of the authors, and we acknowledge the valuable critiques of the reviewers.

Agnes M. Rimando
Stephen O. Duke
U.S. Department of Agriculture
Agritultural Research Service
Natural Products Utilization Research Unit
P.O. Box 8048
University, MS 38677

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.pr001

Natural Products for Pest Management

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.pr001

In Natural Products for Pest Management; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch001

Chapter 1

Natural Products for Pest Management

Agnes M. Rimando and Stephen O. Duke

**Natural Products Utilization Research Unit, Agricultural Research Service,
U.S. Department of Agriculture, P.O. Box 8048, University, MS 38677**

The topic of natural products as pesticides is reviewed, with a discussion of the advantages and disadvantages of adopting a natural product-based strategy for pesticide discovery. Current and past natural product and natural product-based herbicides, insecticides, fungicides, molluscicides, rodenticides, algicides, and nematicides are discussed, giving examples of each. Finally, the potential for genetically engineering synthesis of natural product pesticides into crops is critiqued. Natural products are a unique resource for pest management that we have only begun to utilize.

Historically, the pharmaceutical industry, especially in its early years, was dominated by natural products and natural product-based products, but this cannot be said for the pesticide industry. Some of the earliest pesticides (rotenone, natural pyrethrins, *etc.*) were natural products, but the growth of the pesticide industry into a major component of pest management did not begin until the middle of the last century with the introduction of an ever growing arsenal of synthetic pesticidies, beginning with compounds such as DDT and 2,4-D. Many of the companies involved in pesticide discovery were primarily

U.S. government work. Published 2006 American Chemical Society

pharmaceutical companies. Thus, it was not surprising that a portion of their pesticide discovery effort was based on natural products as templates for new pesticides. Much of this approach involved screening of microbial metabolites, as the pharmaceutical industry has done. Other organisms (terrestrial plants, algae, marine organisms, etc.) have had less emphasis.

There are many reasons why natural products might be good sources of molecules or molecular templates for pesticides. These compounds are the result of coevoluation of the producing organism and its biotic environment. Thus, many of them are defense compounds against other organisms. Natural compounds often have a shorter environmental half life than synthetic compounds, thus reducing potential environmental impact. Isolating and identifying these compounds was an arduous task in the past, but modern instrumentation has simplified and reduced the cost of this process. Natural compound structures are very often not the type of compounds that traditional pesticide chemists generate, so nature is a good source of novel structures. Even though many natural compounds have been described, there are many more to be discovered, and few of the known compounds have been comprehensively examined for all types of potential uses as pesticides. Lastly, the regulatory cost of introducing a natural product as a pesticide is sometimes much less than that of a synthetic chemical.

But, there are some obstacles to a natural product-based pesticide discovery program. One of the down sides of natural products as a resource for pesticide discovery is that many of the more interesting compounds have complex structures with several chiral centers, making their synthesis cost prohibitive. Furthermore, there are increasing legal complexities involving ownership of intellectual property associated with compounds discovered from organisms from some countries (*1*). Evaluation of compounds that are often initially available in only very small amounts is problematic, although miniaturized bioassays have been developed for this. The physicochemical properties of many biologically active natural products is seldom ideal for a pesticide, and the environmental half life is often too short to be effective. For these and other reasons, pesticide discovery efforts based on natural products has never been a substantial part of the overall industrial pesticide discovery effort.

When combinatorial chemistry was introduced, it became very popular to generate huge numbers of new, synthetic compounds to be evaluated in high throughput screens. Natural product-based discovery of both pharmaceuticals and pesticides was further deemphasized. However, this strategy proved not to be cost effective. At least part of the problem may have been that the structural diversity of compounds achieveable with combinatorial chemistry was limited compared to that of natural compounds. An analysis of compounds generated by combinatorial chemistry, versus natural compounds and pharmaceuticals, found that natural compounds and pharmaceuticals have much higher structural

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch001

diversity than compounds produced by combinatorial chemistry that were reported in the literature (*2*).

With the unfulfilled expectations of the combinational chemistry/high throughput screening approach, the pharmaceutical industry is now interested in natural products playing a larger role in their discovery efforts (*3*). Whether this is true for the pesticide industry is not yet apparent. Natural products with biological activity are considered more likely to yield compounds of interest when used as a starting scaffold in combinatorial studies.

Molecular biology has provided a potential for natural products in pest management that does not exist with synthetic pesticides. Natural compounds are synthesized by enzymes that are encoded by genes of the producing organism.. Thus, the organism to be protected can be engineered to make more or new natural pesticides. This approach has tremendous potential.

This chapter provides an overview of the history and current status of natural product use in pest management as an introduction to a group of chapters that cover various aspects and areas of the topic in more detail. Due to length limitations, we cover only the the most important products and a few examples to illustrate concepts.

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch001

Natural Products as Herbicides

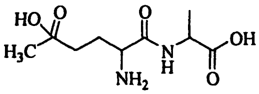
Many natural products are phytotoxic, but few have both the correct physicochemical properties or level of biological activity to be good herbicides. Nevertheless, there are some profound successes in development of herbicides from natural compounds. Furthermore, study of natural phytotoxins has been the source of several new molecular target sites for herbicides. This topic has been covered in more detail in previous reviews (*4-6*).

The most successful natural product used as a herbicide is glufosinate (*7*). Glufosinate is the synthetic version of the natural compound phosphinothricin produced by *Streptomyces* spp.. It is a potent inhibitor of glutamine synthetase and is the only commercial herbicide with this molecular site of action, although there are numerous other natural compounds that inhibit this enzyme. Glufosinate is sold throughout the world under various trade names.

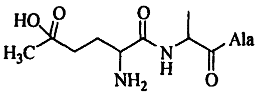
Glufosinate is a relatively fast acting, broad spectrum herbicide with reduced environmental impact, making it ideal for use with transgenic herbicide-resistant crops. The *bar* or *pat* gene, encoding a phosphinothricin acyltranferase from *Streptomyces* spp., has been used to transform crops to generate glufosinate-resistant crops. *Streptomyces* spp. protects itself from this compound with this enzyme. Transgenic glufosinate-resistant cotton, canola, and maize are available in North America (*8*).

Streptomyces spp. makes bialaphos as an inactive precursor of phosphinothricin. When applied to weeds, it is bioactivated by metabolic conversion to phosphinothricin (*9*). The bioactivation process is not equally efficient in all plants, making bialaphos more selective than is phosphinothricin. It is manufactured as a herbicide in Japan by fermentation.

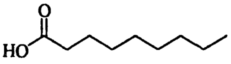
The only other pure natural organic compound that is sold as a herbicide is pelargonic acid (nonoic acid). It has limited use as a postemergence contact herbicide (*e.g., 10*). Mixtures of other fatty acids are also available as herbicides for organic farmers, but pure pelargonic acid is much more potent. In the U.S. there is an exemption from the requirement of a tolerance (maximum permissible level) for residues of pelargonic acid in food commodities when used as a herbicide (*11*). Its mode of action has been reported to involve both membrane disruption and light-enhanced peroxidation of membrane lipids (*12*).



Phosphinothricin (Glufosinate)



Bialaphos

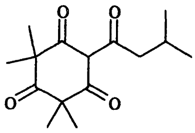


Pelargonic acid

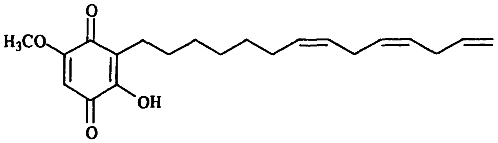
Mixtures of organic natural products, such as a mixture of vinegar, lemon extract, and clove oil, are sold for organic weed management. Maize gluten meal is also used as a herbicide and fertilizer (*13*). Its herbicidal activity is due to phytotoxic dipeptides (*14*) and a pentapeptide (*15*). This product has limited use in organic crops and turf.

Other successful herbicides are analogues of natural products, but in only a few cases have those who have discovered them revealed that their discovery was based on the natural product analogue. The most important of these are the triketone herbicides, such as sulcotrione, whose discovery was based on the phytotoxicity of an alleolochemical, leptospermone, found in the the bottlebrush plant (*Callistemon* spp.) (*16*). Leptospermone is also a major component of the essential oil of New Zealand manuka (*Leptospermum scoparium*) (*17*). The triketones inhibit hydroxyphenyl pyruvate dioxygenase (HPPD), an enzyme responsible for synthesis of plastoquinone (PQ) in plants (*18*). PQ is a cofactor for the first enzyme committed to carotenoid synthesis, phytoene desaturase. Thus, inhibition of PQ synthesis by inhibiting HPPD activity leads to white leaf tissue and loss of chloroplast development. Several other phytotoxic natural products, such as sorgoleone and usnic acid are good inhibitors of HPPD (*19*), however, whether this accounts for their phytotoxicity is unknown. Sorgoleone is also an analogue of plastoquinone, and inhibits photosystem II of photosynthesis by binding the plastoquinone binding site of the D1 protein (*20*).

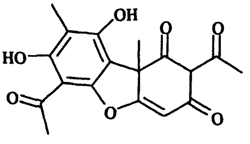
Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch001



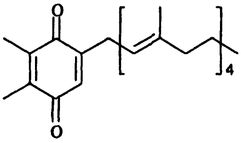
Leptospermone



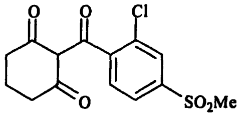
Sorgoleone



Usnic acid



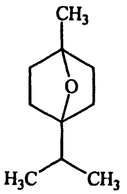
Plastoquinone



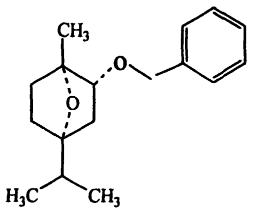
Sulcotrione

Several past or present commercial herbicides are close analogues of natural products, but whether their discovery was part of a natural product-based discovery effort was never divulged. These include cinmethylin, an analogue of the monoterpene 1,4-cineole, a known phytotoxin (*21*). Several commercial auxinic herbicides, such as dicamba, are benzoic acid analogues. Cantharidin is a more potent phytotoxin than its commercial herbicide analogue endothall (unpublished work of S.O. Duke and J. Basja).

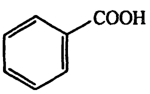
There are many patents on natural phytotoxins and their derivatives as herbicides. For example, several companies have patents on analogues of the microbial metabolite hydantocidin as a herbicide (e.g., *22*). After metabolic phosphorylation, hydantocidin is a potent inhibitor of adenylosuccinate synthetase (*23*), an enzyme involved in nucleotide biosynthesis. As with synthetic compounds only a small fraction of patented herbicides have made it to the marketplace.



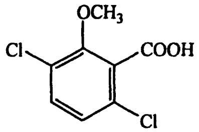
1,4-Cineole



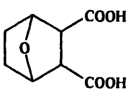
Cinmethylin



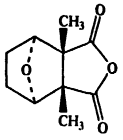
Benzoic acid



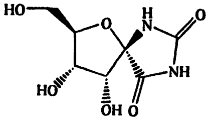
Dicamba



Endothall



Cantharidin



Hydantocidin

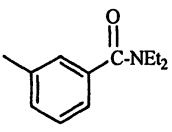
Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch001

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch001

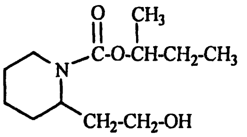
DEET (*N,N*-diethyl-*m*-toluamide) has been the only repellent ingredient endorsed by the U.S. Centers for Disease Control and Prevention (CDC) to effectively repel mosquitoes for the last 50 years until April 28, 2005 when the CDC released new guidance on effective mosquito repellents available in the U.S., which included the addition of two new active ingredients: picaridin (also known as KBR3023) and *p*-menthane-3,8-diol (also known as oil of lemon eucalyptus or PMD) (http://www.cdc.gov/od/oc/media/pressrel/r050428.htm). The endorsement of PMD, a plant-based mosquito repellent, is a recognition of natural products as an equally important source of repellents. Four isomers of PMD make up the natural product from the oil of *Eucalyptus citriodora*. These isomers, obtained synthetically, were equally repellent to *Anopheles gambiae* (*24*).

The efficacy of four synthetic and eight natural product-based repellents against *Aedes albopictus*, *Culex nigripalpus*, and *Ochlerotatus triseriatus* was evaluated in a laboratory test. Autan (10% picaridin), Bite Blocker (2% soybean oil), Off (15% DEET), and Repel (26% PMD) were the most repellent, preventing biting for ≥7.2 h when the estimated mean protection time responses were averaged for all three mosquito species (*25*).

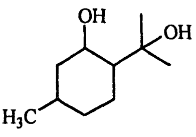
The essential oils of *Eucalyptus globulus, Lavandula officinalis, Rosmarinus officinalis, and Thymus vulgaris* are also effective repellents (*26*). In an assay, thyme oil was the most effective of the four oils in repelling adult mosquitoes on hairless mice. Analysis of the oil by gas chromatography-mass spectrometry (GC-MS) showed the predominance of five monoterpenes, of which α-terpinene was the most potent. A spray-type solution containing 2% α-terpinene showed stronger repellent activity than DEET against *Culex pipiens pallens*. Extracts from the medicinal plant *Myroxylon balsamum* were assayed for larvicidal on third instar *Aedes aegypti* larvae. The sesquiterpene nerolidol was found to be the active constituent in the hexane extract, and the sesquiterpenes were more active than the monoterpenes and phenylpropanoids tested (*27*). The terpenoids spathulenol, intermedeol, and callicarpenol from the foliage of *Callicarpa americana* are potent mosquito repellents (*28*).



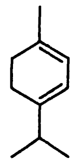
DEET



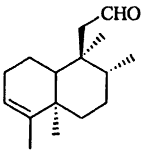
Picaridin



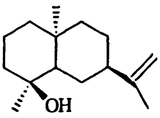
p-Menthane-3,8-diol



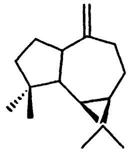
α-Terpinene



Callicarpenal

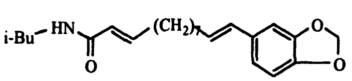


Intermedeol

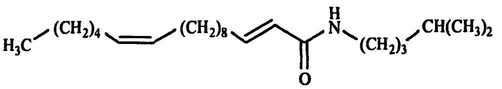


Spathulenol

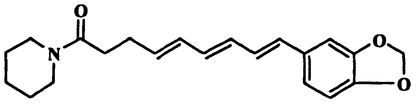
Mosquito repellent compounds from the genus *Piper* have also been reported. Three amides pipgulzarine, pipzorine, and piptigrine, obtained from the seeds of *Piper nigrum* exhibited toxicity against the fourth instar larvae of *Aedes aegypti* (*29, 30*). A piperidine amide, pipernonaline, was also identified as the larvicidal component from the methanol extract of *Piper longum* fruits (*31*).



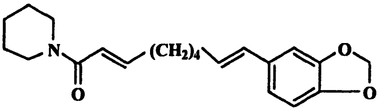
Pipgulzarine



Pipzorine

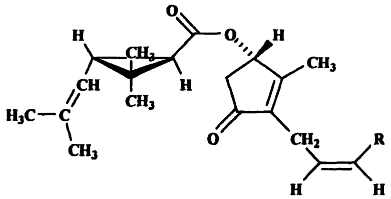


Piptigrine

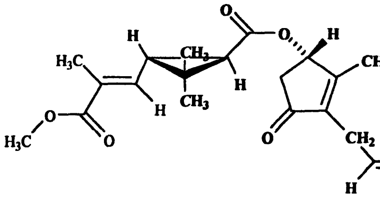


Pipernonaline

Certain species of *Chrysanthemum* are sources of insecticidal compounds collectively known as pyrethrins, which have been used for centuries for the control of several species of insects. The industrial production of pyrethrins is based on their extraction from *Chrysanthemum cinerariaefolium.* Pyrethrum extracts are standardized for the content of the two major constituents pyrethrin-I and -II, as well as four other minor constituents, cinerin-I and -II, and jasmolin-I and –II (*32*). Different biotechnological approaches such as callus, shoot and root, and plant cell suspension cultures, bioconversion of precursors by means of enzymic synthesis, or use of genetically engineered microorganisms to produce pyrethrins have been tried but seem to be of limited application (*33*). One of the most successful classes of synthetic insecticides are the synthetic pyrethins, which are analogues of natural pyrethroids.



Pyrethrin I, R = $CH=CH_2$
Jasmolin I, R = CH_2CH_3
Cinerin I, R = CH_3



Pyrethrin II, R = $CH=CH_2$
Jasmolin II, R = CH_2CH_3
Cinerin II, R = CH_3

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch001

The mechanisms of its insect repellency are not clearly understood. However, the identification of a protein from *Anopheles gambiae*, AgOr1, a female-specific member of a family of putative odorant receptors that respond to a component of human sweat provides a clue to the possible site of action for compounds that activate or block receptors of this type. These compounds could act as attractants or repellents (*34*).

The neem tree, *Azadirachta indica* A. Jussieu 1830 (Meliaceae), has been a source of numerous compounds with a wide range of insectidical properties. The major constituent is azadirachtin, a complex triterpenoid isolated from the seeds. It is a potent insect antifeedant, growth disruptant and possesses anti-malaria activity. Azadirachtin affects other insect biological processes including suspension of molting, death of larvae and pupae, and sterility of emerged adults. Several other insecticidal compounds of the limonoid group of triterpenes have been isolated from the fruit and leaves, which were toxic to the 4th-instar larvae of *Anopheles stephensi*. The LC_{50} values ranged from 2.8 to 150 ppm (*35-37*).

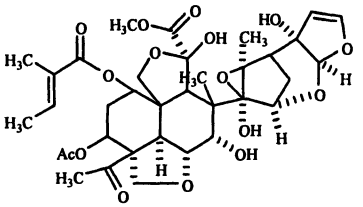
A possible target for the activity of the neem limonoids are the gut enzymes. Rice leaffolder (*Cnaphalocrocis medinalis*) larvae fed with a diet of rice leaves treated with the limonoids azadirachtin, salannin, deacetylgedunin, gedunin, 17-hydroxyazadiradione, and deaceytlnimbin exhibited a significant reduction in activities of the gut tissue enzymes acid phosphatases, alkaline phosphatases, and ATPases (*38*). It is also suggested that azadirachtin affects hormones and proteins involved in reproduction (*39*). In another study changes in the protein metabolism of *Spodoptera litura* fed with an artificial diet containing 1 ppm azadirachtin from the 4th instar larvae until pupation was shown. Ten proteins were significantly affected by azadirachtin. One of these proteins, identified with peptide mass fingerprinting using MALDI-TOF-MS after in-gel trypsin digestion, appear to function as an ecdysone receptor that regulates insect development and reproduction (*40*).

Means to control the Formosan subterranean termite [*Coptotermes formosanus* (Shiraki) Fam. Rhinotermitidae] (FST) with the use of natural termiticides is a growing area of research. Termites are a major problem in the warm and humid Southeast region of the United States and cause an estimated $1 billion in damage annually. In April 1998, “Operation Full Stop,” a national campaign against the FST was launched. Some termiticidal natural products have been discovered, one being vetiver oil. Nootkatone, a major constituent in vetiver oil, affected FST and their protozoa, acting as arrestant, repellent and feeding deterrent. Its derivatives, 1,10-dihydronootkatone and tetrahydronootkatone were found to be more effective in repellency and acute toxicity testing (*41*). T-muurolol isolated from the extracts of the leaves of *Calocedrus formosana*, an endemic tree species in Taiwan known for its natural decay, reportedly caused 100% mortality of FST at a dosage of 5 mg/g after 14 d (*42*). Apigenin was found to be the most toxic to the FST fed at 50 µg/primary

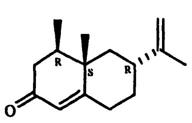
Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch001

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch001

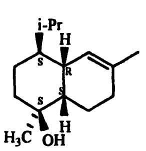
reproductive pair, while biochanin-A was the most effective in reducing fecundity among five flavonoids tested (*42*). *cis*-Nerol effected morphological abnormalities in the exoskeleton of the FST after 2 hr exposure, and appeared to cause damage to cell membranes (*42*). Bioassay-guided fractionation of the EtOAc fraction of the methanol extract of *Cryptomeria japonica* yielded cryptomerione, cubenol, epicubenol, cubebol, and 12-hydroxy-6,7-secoabieta-8,11,13-triene-6,7-dial as the compounds with potent activity against the FST, along with the known termiticidal substances T-cadinol, 16-phyllocladanol, sandaracopimarinol, and β-eudesmol (*45*). There's a continuing effort to discover natural termiticides.



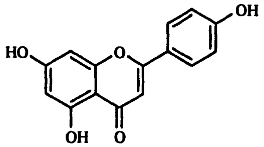
Azadirachtin



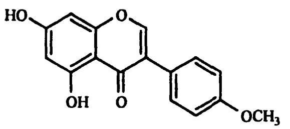
Nootkatone



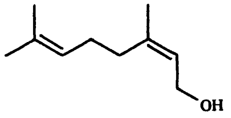
T-Muurolol



Apigenin

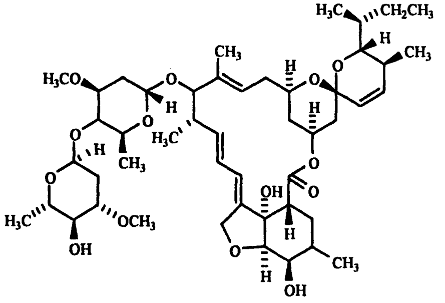


Biochanin A

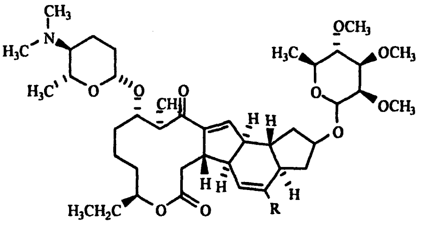


cis-Nerol

Microorganisms have proven to be a rich source of potent natural insecticidal compounds, exemplified by spinosyns which are a family of macrocyclic lactones derived from species of the actinomycete bacterium *Saccharopolyspora spinosa*. Spinosyns are active on a wide variety of insect pests, especially the lepidopterans and dipterans. A mixture of the two most abundant spinosyns (Spinosyn A and D) displays the efficacy of a synthetic insecticide. Studies on the mode of action of spinosyns suggest that both nicotinic and gamma-aminobutyric acid receptor functions are altered. Several spinosyns have been isolated and spinosoids (semi-synthetic analogs) synthesized. Quantitative structure activity relationships of spinosyns have been reviewed (*46*). Alteration of the function of δ-aminobutyric acid channels and nicotinic acetylcholine receptors is suggested from several studies as the mode of action of spinosad (*47*). Avermectins are another group of insecticidal compounds derived from microorganisms, isolated from the mycelia of the soil microorganism *Streptomyces avermitilis*. A 1% emulsion of Abamectin (a mixture of avermectins containing not more than 80% avermectin B1a and not less than 20% avermectin B1b) is toxic to a wide variety of insects: caterpillars, leaf-miner fly, whitefly, aphids, as well as Colorado potato beetle (*48*). Avermectins are also efficacious and safe for use as anti-parasitic agents. Omura (*49*) provided a review on the mode of action of avermectin. Screening of fungal culture extracts using a GABA-receptor binding assay led to the isolation of two alkaloids from *Aspergillus terreus*: alantrypinone and serantrypinone (*50*). The mycotoxins penitrems A-D and F from the mycelium of *Penicillium crustosum* showed convulsive and insecticidal activities against the hemipteran *Oncopeltus fasciatus* Dallas and the dipteran *Ceratitis capitata* Wiedemann. Reduction in the fecundity and fertility of the surviving *C. capitata* females were also observed (*51*). The potential of insecticidal fungal metabolites for development into commercial products has been reviewed (*52*).



Avermectin B1a



Spinosyn-A, R = H; -D, R=CH_3

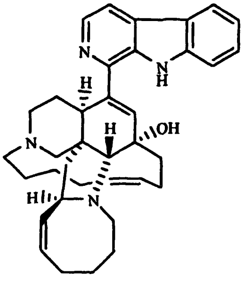
Insecticidal compounds have also been isolated from marine organisms (*53*). The manzamine alkaloids obtained from marine sponge, manzamine A, *ent*-8-hydroxymanzamine A, and *ent*-hydroxymanzamine F were toxic to the western corn rootworm (*Diabrotica virgifera*). Manzamine A was also found to be toxic against the western tarnished plant bug (*Lygus hesperus*) (*53*). Insecticidal compounds from some Indo-Pacific marine invertebrates were included in a review (*54*).

Natural products have been shown to effectively control several other kinds of insect pests. Two isomers of nepetalactone [2-(2-hydroxy-1-methylethenyl)-5-methyl-cyclopentanecarboxylic acid delta lactone], the major constituent of catnip oil, and elemol from the oil of osage orange were shown to be as good or better than DEET at repelling house flies, *Musca domestica*, and American cockroaches, *Periplaneta Americana* (*55*). The *E,Z*-nepetalactone isomer is more repellent to cockroaches than the dominant isomer, *Z,E*-nepetalactone (*56*). Sargachromenol, sargahydroquinoic acid, and sargaquinoic acid from the aerial parts of *Roldana barba-johannis* and their corresponding acetylated and methylated derivatives were insecticidal to the Fall Armyworm (*Spodoptera frugiperda*), an insect pest of corn (*57*). Stilbenes from the methanol extract of the bark of *Yucca periculosa* were shown to have growth regulatory effect on the

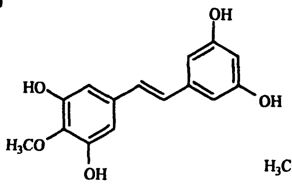
Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch001

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch001

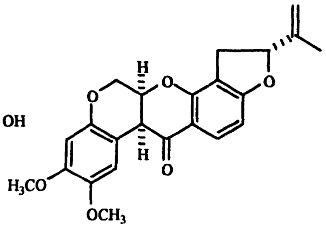
Fall Army worm. 3,3',5,5'-Tetrahydroxy-4-methoxystilbene demonstrated the most significant effects at 3 μg/g in diets. The stilbenes are thought to interfere with the processes of sclerotization and moulting (*58*). Camphor extracted from *Cinnamonum camphora* was highly effective for the control of the long-horned beetles (*Anoplophora chinensis* and *Nadezhdiella cantori*) in field experiments (*59*). The toxicity of *Hippocratea excelsa* root cortex has been previously demonstrated against the stored grain pest *Sitophilus zeamais*. The triterpenoid pristimerin and a mixture of sesquiterpene evoninoate alkaloids isolated from this plant strongly reduced the insect feeding capacity (*60*). *Lantana camara* and *Tephrosia vogelii* were reported to have activity against the corn weevil *Sitophilus zeamais* (*61*). The main insecticidal constituents of *T. vogelii* are rotenoids (*62*), which is also the group of insectidal compounds in *Derris* species, a natural pesticide that has been used for several years.



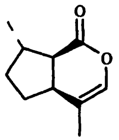
Manzamine A



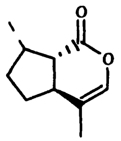
3,3',5,5'-Tetrahydroxy-4-methoxystilbene



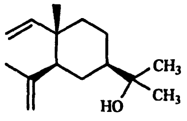
Rotenone



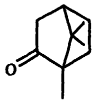
Z,E-Nepetalactone



E,Z-Nepetalactone



Elemol

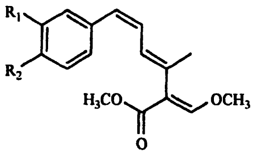


Camphor

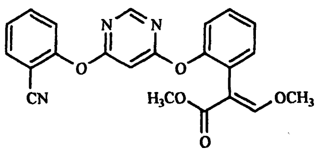
The growing number of studies on the use of natural products for the control of insect pests reflects the quest for substitutes to existing insect control agents. Their application is diverse; an example is citronella-treated carton which was shown to effectively deter infectation by red flour beetles (*63*). Natural insecticides may also be incorporated in mosquito nets (*64*). The endorsement of PMD is encouraging; it can be expected that natural compounds will become a larger part of today's insectide industry.

Natural Products as Fungicides

One of the most important fungicides on the market is the strobilurin group of chemicals, a member of a larger group of quinone outside inhibitors (QoI). QoI act at the Qo center of the cytochrome bc1-complex. The strobilurin fungicides was named with reference to the wood-rotting mushroom fungi, *Strobilurus tenacellus*, from which the first compound in this group was isolated. Several synthetic analogs with improved efficacy and stability have been prepared. This includes azoxystrobin, the world's biggest selling fungicide (*65*). Resistance to the commercial, synthetic strobilurin fungicides has been documented and is becoming widespread (*66*). The developing resistance to currently used fungicides triggers the search for new agents, and natural products is one resource pool being tapped.

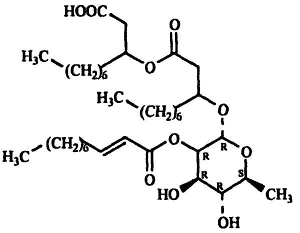


Strobilurin A, $R_1 = R_2 = H$
Strobilurin B, $R_1 = OCH_3$, $R_2 = Cl$

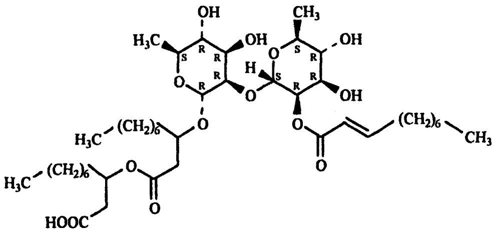


Azoxystrobin

Rhamnolipids, metabolites of *Pseudomonas aeruginosa*, show promise as broad-spectrum fungicides having been shown to be highly effective against several economically important fungi such as *Cercospora kikuchii*, *Cladosporium cucumerinum*, *Colletotrichum orbiculare*, *Cylindrocarpon destructans*, *Magnaporthe grisea*, *Phytophthora capsici*, *P. cryptogea*, *Plasmopara lactucae-radicis*, *Pythium aphanidermatum and Rhizoctonia solani.* Rhamnolipids have surfactant properties, killing zoospores by rupturing the plasma membrane (*67-69*). On March 31, 2004, the U.S. Environmental Protection Agency issued a rule concerning the establishment of an exemption from the requirement of a tolerance for residues of rhamnolipids on all food commodities when applied/used as a fungicide (*70*).



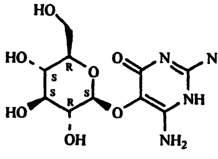
Rhamnolipid A



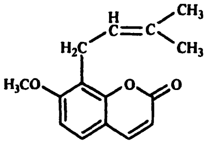
Rhamnolipid B

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch001

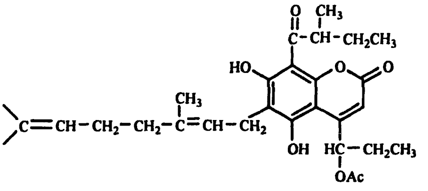
Other natural fungicidal compounds have been reported that look promising. One is vicine, isolated from mature seeds of *Vicia faba.* Vicine was shown to be signicantly fungistatic to *Fusarium culmorum* and *Alternaria alternate in vitro* at 5 ppm, and to *Cladosporium herbarum* and *Botrytis cinerea* at 0.5 ppm. The effect of vicine was comparable with those of standard fungicides used in the assay and with the plant saponin digitonin. Vicine was also found to have insecticidal properties. Additionally, the effective concentration of vicine in the *in vitro* assay was significantly lower than its concentration in the seeds and pods (*71*). Osthol, a coumarin that have been isolated in a few plant species, was found to have a wide spectrum of activity, being inhibitory to several fungi including *Rhizoctonia solani, Colletotrichum musae, Rhizoctonia solani, Phytophthora capsici, Colletotrichum gloesporioides, Botrytis cinerea, Sclerotinia sclerotiorum, Fusarium graminearum, Macrophoma kawatsukai, Fusarium oxysporum* f. sp. *niveum and F. oxysporum* f. sp. *vasinfectum*, with EC_{50} values ragning from 21.15 μg/mL to 61.61 μg/mL. Studies on its mechanism of action showed it inhibited spore germination. *F. graminearum* treated with 100 μg/mL for 24 h showed an increase in content of soluble proteins, chitin, and chitinase activity (*72*). Surangin B, a coumarin isolated from *Mammea longifolia*, strongly inhibited the mycelial growth of *Rhizoctonia solani* (IC_{50} = 3.8 μM) and *Botrytis cinerea* (IC_{50} = 11.2 μM), and reduced levels of spore germination in *Fusarium oxysporum* (IC_{50} = 2.3 μM) and *Botrytis cinerea* (IC_{50} = 1.4 μM). Surangin B is an electron transport inhibitor (*73*).



Vicine



Osthol

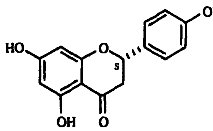


Surangin B

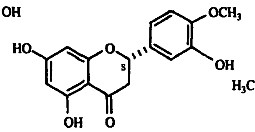
Phytoalexins, defense compounds produced by plants in response to fungal infection and other stresses, can also be molecular templates or leads as fungicides. Infection of *Citrus sinensis* fruit with *Phytophthora citrophthora* caused an increase in the levels of the polymethoxyflavones sinensetin, nobiletin, tangeretin, and heptamethoxyflavone. The levels of the flavanones hesperidin and isonaringin decreased but those of their corresponding aglycones, hesperetin and naringenin, increased suggesting the hydrolysis of the flavanone glycosides. *In vitro* studies demonstrated these compounds to be antifungal agents, with naringenin and hesperetin being the most active (*74*). The activity of the alfalfa phytoalexin medicarpin against eight phytopahtogenic fungi was tested *in vitro* together with its four biosynthetic intermediates, vestitone, 2'-hydroxyformononetin, formononetin and daidzein. The fungicidal activities of

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch001

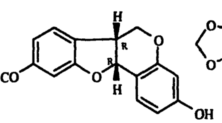
these phytoalexins differed with the test organism (*75*). Medicarpin, homopterocarpin, isoliquiritigenin, liquiritigenin, and 4,2',5'-trihydroxy-chalcone isolated from the heartwood extracts of *Platymiscium yucatanum*, a tropical wood highly resistant to the fungi *Lenzites trabea* and *Coriolus versicolor*, showed varying degrees of inhibitory activity against these two orgnanisms *in vitro*. Medicarpin was the most active and was more inhibitory than phenol against *C. versicolor* (*76*). Medicarpin and maackiain inhbited *Fusarium oxysporum* f. sp. *ciceri*, the pathogen causing wilt, at concentrations similar to those recorded in wilt-resistant chickpea roots, indicating that these pterocarpans are fundamental components of the resistance mechanism of chickpeas to wilt (*77*).



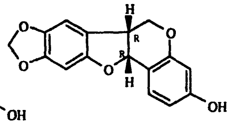
Naringenin



Hesperetin



Medicarpin



Maackiain

Numerous other fungicidal constituents from natural sources have been reported with activity towards specific fungi. To find a natural product that is fungicidal to a wide range of organisms will find broad application, and there is continuing effort to achieve this goal.

Miscellaneous Natural Product Pesticides

Pesticides other than herbicides, insecticides, and fungicides have relatively small market products. Thus, there is less financial incentive for discovery and development of these materials. These products include molluscicides, rodenticides, and algicides. The relatively low cost of regulatory approval of some natural products should make natural products the approach of choice in discovery of these pesticides.

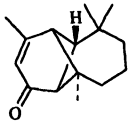
We are aware of no commercial natural, organic products for mollusk management. However, there have been published reports of natural products from plants, such as vulgarone B (*78*) and hederagenin (*79*), being effective molluscicides.

The most commonly used rodenticides are synthetic blood anticoagulants, however strychnine, an alkaloid extracted from *Strychnos nux vomica*, is commercially available for use by licensed pest control personnel (*80*). Before synthetic anticoagulants were discovered, several natural product-based rodenticides were used, such as red squill or scilliroside, a botanical compound extracted from the bulb of the Mediterranean squill plant *(Urginea maritima*) (*81*).

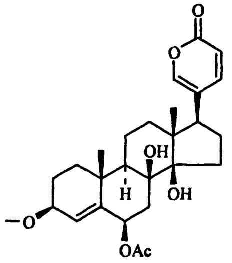
Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch001

Some natural nematocides, such as a crude preparation of cardenolides from milkweed seed meal, are available, but we are aware of no pure, natural compounds being sold as a nematicide. There are numerous published studies on natural compounds being toxic to nematodes (*e.g.*, *82*).

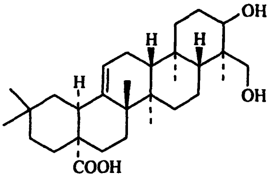
Few algicides are commerccially available, and none of these products are natural products. Work by Schrader in our laboratory has resulted in the discovery that 9,10-anthroquinone is a good selective algicide for cyanobacterial algae (*83*). Improvement of the physicochemical properties of 9,10-anthroquinone to produce 2-[methylamino-N-(1'-methylethyl)]-9,10-anthraquinone mono-phosphate, resulted in a more effective algicide in a field environment (*84*).



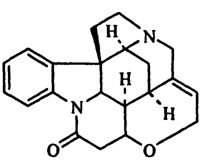
Vulgarone B



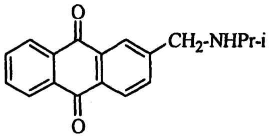
Scilliroside



Hederagenin



Strychnine



9,10-Anthroquinone derivative

Molecular Biology and Natural Products for Pest Management

Natural products are synthesized by enzymes that are encoded by genes of the producing organisms. Through transgenic technologies new genes for crop protection compounds can be introduced into crops or biocontrol agents, or the expression of existing genes that encode a pathway to a protectant compound that is not produced in adequate quantities can be enchanced. The only commercial success with this approach so far has been with transgenes encoding various proteinaceous toxins of *Bacillis thuriengensis*. Cotton and maize using such transgenes for insect control are widely used in North America and a growing number of countries from other parts of the world. There is considerable research to use this approach to generate other pesticidal compounds in crops. Much of this effort is focused on one gene approaches to produce pesticidal

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch001

peptides that target insects or plant pathogens (*e.g.*, *85-88*). Genes for peptide-based phytotoxins have been put into microbial biocontrol agents for weed control (*89*).

Manipulating the enzymology of the synthesis of non-peptide pesticidal compounds is in its infancy. Modication of terpenoid synthesis in potato for insect control is one example of such strategy (*90*). Our laboratory is involved in an approach to improve allelopathy in *Sorghum* spp. by transgenically enhancing sorgoleone production (*91, 92*). Much of the reseach on transgenically enchancing or changing secondary metabolism of plants has been to produce high value compounds such as pharmaceuticals. Manipulation of secondary metabolism can cause unpredicatble effects. This approach will require much more research to be successful.

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch001

References

1. Kate, K.; Laird, S. A. *The Commercial Use of Biodiversity*; Earthscan Publications: London, 1999; 398 pp.
2. Feher, M.; Schmidt, J. M. *J. Chem. Inf. Comput. Sci.* **2003**, *43*, 218-227.
3. Rouhi, A. M. *Chem. Eng. News* **2003**, *81*, 77-78, 86, 88-91.
4. Duke, S. O.; Dayan, F. E.; Rimando, A. M. In *Herbicides and Their Mechanisms of Action*; Cobb, A.H.; Kirkwood, R.C., Eds.; Sheffield Academic Press: UK, 2000; pp. 105-133.
6. Duke, S. O.; Dayan F. E.; Rimando, A. M.; Schrader, K. K.; Aliotta, G.; Oliva, A.; Romagni, J. G. *Weed Sci.* **2002**, *50*, 138-151.
7. Lydon, J.; Duke, S. O. In *Plant Amino Acids: Biochemistry and Biotechnology*; Singh, B.K., Ed.; Marcel Dekker, Inc.: New York, NY, 1999; pp. 445-464.
8. Duke, S. O. *Pest Manag. Sci.* **2005**, *61*, 211-218.
9. Wild, A.; Ziegler, C. *Z. Naturforch.* **1989**, *44c*, 97-102.
10. Bradley, K. W.; Hagood, E. S. *Weed Technol.* **2002**, *16*, 164-170.
11. Environmental Protection Agency, USA. Federal Register **1997**, *62*, 28361-28364.
12. Lederer, B.; Fujimori, T.; Tsujino, Y.; Wakabayashi, K.; Böger, P. *Pestic. Biochem. Physiol.* **2004**, *80*, 151-156.
13. Gardner, D. S.; Christians, N. E.; Bingaman, B. R. *Crop Sci.* **1997**, *37*, 1875-1877.
14. Liu, D. L.; Christians, N. E. *J. Plant Growth Regul.* **1994**, *13*, 227-230.
15. Liu, D. L.; Christians, N. E. *J. Plant Growth Regul.* **1996**, *15*, 13-17.
16. Lee, D. L.; Prisbylla, M. P.; Cromartie, T. H.; Dagarin, D. P.; Howard, S. E.; Provan, W. M.; Ellis, M. K. *Weed Sci.* **1997**, *45*, 601-609.

17. Douglas, M. H.; van Klink, J. W.; Smallfield, B. M., Perry, N. B.; Anderson, R. E.; Johnstone, P.; Weavers, R. T. *Phytochemistry* **2004**, *65*, 1255-1264.
18. Moran, G. R. *Arch. Biochem. Biophys.* **2005**, *433*, 117-128.
19. Meazza, G.; Scheffler, B. E.; Tellez, M. R.; Rimando, A. M.; Romagni, J. G.; Duke, S. O.; Nanayakkara, D.; Khan, I. A.; Abourashed, E. A.; Dayan, F. E. *Phytochemistry* **2002**, *60*, 281-288.
20. Streibig, J. C.; Dayan, F. E.; Rimando, A. M.; Duke, S. O. *Pestic. Sci.* **1999**, *55*, 137-146.
21. Romagni, J. G.; Allen, S. N.; Dayan, F. E. *J. Chem. Ecol.* **2000**, *26*, 303-313.
22. Harrington, P. M.; Jung, M. E. U.S. Patent 5354868, 1994.
23. Siehl, D. L.; Subramanian, M. V.; Walters, E. W.; Lee, S. F.; Anderson, R. J.; Toschi, A. G. *Plant Physiol.* **1996**, *110*, 753-768.
24. Barasa, S. S.; Ndiege, I. O.; Lwande, W.; Hassanali, A. *J. Med. Entomol.* **2002**, *39*, 736-741.
25. Barnard, D. R.; Xue, R.-D. *J. Med. Entomol.* **2004**, *41*, 726-730.
26. Choi, W.-S.; Park, B.-S.; Ku, S.-K.; Lee, S.-E. *J. Am. Mosq. Control Assoc.* **2002**, *18*, 348-351.
27. Simas, N. K.; Lima, E. C.; Conceicao, S. R.; Kuster, R. M.; Martins de Oliveira Filho, A.; Lage, C. L. Sa. *Quim. Nova* **2004**, *27*, 46-49.
28. Cantrell, C. L. ; Klun, J. A. ; Bryson, C. T. ; Kobaisy, M. ; Duke, S. O. *J. Agric. Food Chem.* **2005**, *In press.*
29. Siddiqui, B. S.; Gulzar, T.; Begum, S.; Rasheed, M.; Sattar, F. A.; Afshan, F. *Helv. Chim. Acta* **2003**, *86*, 2760-2767.
30. Siddiqui, B. S.; Gulzar, T.; Begum, S.; Afshan, F. *Nat. Prod. Res.* **2004**, *18*, 473-477.
31. Yang, Y.-C.; Lee, S.-G.; Lee, H.-K.; Kim, M.-K.; Lee, S.-H.; Lee, H.-S. *J. Agric. Food Chem.* **2002**, *50*, 3765-3767.
32. Dickinson, C. M. *JAOAC* **1982**, *65*, 921-926.
33. Hitmi, A.; Coudret, A.; Barthomeuf, C. *Crit. Rev. Plant Sci.* **2000**, *19*, 69-89.
34. Hallem, E. A; Nicole, F. A; Zwiebel, L. J; Carlson, J. R. *Nature* **2004**, *427*, 212-213.
35. Tariq, R. M.; Naqvi, S. N. H.; Siddiqui, B. S.; Rasheed, M.; Faizi, S.; Aslam, M.; Zafar, S. M. N. *Internat. J. Biol. Biotech.* **2004**, *1*, 83-89.
36. Siddiqui, B. S.; Afshan, F.; Faizi, S.; Naqvi, S. N.; Tariq, R. M. *J. Nat. Prod.* **2002**, *65*, 1216-1218.
37. Siddiqui, B.S.; Afshan, F.; Gulzar, T.; Sultana, R.; Naqvi, S. N.-H.; Tariq, R.M. *Chem. Pharm. Bull.* **2003b**, *51*, 415-417.
38. Nathan, S. S.; Kalaivani, K.; Murugan, K.; Chung, P.G. *Pestic. Biochem. Physiol.* **2005**, *8*, 113-122.

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch001

39. Medina, P.; Budia, F.; del Estal, P.; Vinuela, E. *J. Econ. Entomol.* **2004**, *97*, 43-50.
40. Huang, Z.; Shi, P.; Dai, J.; Du, J. *Pestic. Biochem. Physiol.* **2004**, *80*, 85-93.
41. Ibrahim, S. A.; Henderson, G.; Zhu, B. C. R.; Fei, H.; Laine, R.A. *J. Econ. Entomol.* **2004**, *97*, 102-111.
42. Cheng, S.-S.; Wu, C.-L.; Chang, H.-T.; Kao, Y.-T.; Chang, S.-T. *J. Chem. Ecol.* **2004**, *30*, 1957-1967.
43. Boue, S. M.; Raina, A. K. *J. Chem. Ecol.* **2003**, *29*, 2575-2584.
44. Zhu, B. C. R.; Henderson, G.; Fei, H.; Ying, X.; Laine, R. A. *J. Entomol. Sci.* **2003**, *38*, 225-233.
45. Arihara, S.; Umeyama, A.; Bando, S.; Kobuke, S.; Imoto, S.; Ono, M.; Yoshikawa, K.; Amita, K.; Hashimoto, S. *Mokuzai Gakkaishi* **2004**, *50*, 413-421.
46. Sparks, T. C.; Crouse, G. D.; Durst, G. *Pest Manag. Sci.* **2001**, *57*, 896-905.
47. Sarfraz, M.; Dosdall, L. M.; Keddie, B. A. *Outlooks Pest Manag.* **2005**, *16*, 78-84.
48. Petkov, B.; Atanasova, G.; Videnova, E.; Markovski, I.; Aleksieva, V. *Godishnik na Sofiiskiya Universitet Sv.Kliment Okhridski. Biologicheski Fakultet. Kniga 1: Zoologiya* **2000**, *92*, 25-31.
49. Omura, S. In *Macrolide Antibiotics*; Omura, S., Ed.; Elsevier Inc.: New York, NY, 2002; pp 571-576.
50. Kuriyama, T.; Kakemoto, E.; Takahashi, N.; Imamura, K.; Oyama, K.; Suzuki, E.; Harimaya, K.; Yaguchi, T.; Ozoe, Y. *J. Agric. Food Chem.* **2004**, *52*, 3884-3887.
51. Gonzalez, M. C.; Lull, C.; Moya, P.; Ayala, I.; Primo, J.; Yufera, E. P. *J. Agric. Food Chem.* **2003**, *5*, 2156-2160.
52. Anke, H.; Sterner, O. In *Mycota*; Osiewacz, H. D., Ed.; Springer-Verlag: Berlin, Germany, 2002; pp 109-127.
53. Peng, J.; Shen, X.; El Sayed, K. A; Dunbar, D. C.; Perry, T. L.; Wilkins, S. P.; Hamann, M. T.; Bobzin, S.; Huesing, J.; Camp, R.; Prinsen, M.; Krupa, D.; Wideman, M. A. *J. Agric. Food Chem.* **2003**, *51*, 2246-52.
54. Edrada, R. A.; Wray, V.; Handayani, D.; Schupp, P.; Balbin-Oliveros, M.; Proksch, P. In *Studies in Natural Products Chemistry*; Atta Ur Rahman, Ed.; Bioactive Natural Products (Part B) 21; Elsevier Science B. V.: Amsterdam, The Netherlands, 2000; pp 251-292.
55. Schultz, G.; Simbro, E.; Belden, J.; Zhu, J.; Coats, J. *Environ. Entomol.* **2004**, *33*, 1562-1569.
56. Peterson, C. J.; Nemetz, L. T.; Jones, L. M.; Coats, J. R. *J. Econ. Entomol.* **2002**, 95, 377-380.
57. Cespedes, C. L.; Torres, P.; Marin, J. C.; Arciniegas, A.; Romo de Vivar, A.; Perez-Castorena, A. L.; Aranda, E. *Phytochemistry* **2004**, *65*, 1963-1975.

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch001

58. Torres, P.; Avila, J. G.; Romo de Vivar, A.; Garcia, A. M.; Marin, J. C.; Aranda, E.; Cespedes, C. L. *Phytochemistry* **2003**, *64*, 463-473.
59. Li, B. *Zhiwu Baohu Xuebao* **2003**, 30, 166-170.
60. Reyes-Chilpa, R.; Jimenez-Estrada, M.; Cristobal-Telesforo, E.; Torres-Colin, L.; Villavicencio, M. A.; Perez-Escandon, B. E.; Mercado-Gonzalez, R. *Econ. Bot.* **2003**, *57*, 54-64.
61. Ogendo, J. O.; Belmain, S. R.; Deng, A. L.; Walker, D. J. *Acta Hort. (ISHS)* **2005**, *679*, 137-143.
62. Zhang, Y.; Xu, H.; Huang, J.; Chin, S. *Tianran Chanwu Yanjiu Yu Kaifa* **2000**, *12*, 6-12.
63. Wong, K. K. Y.; Signal, F. A.; Campion, S. H.; Motion, R. L. *Appita Ann. Conf. Proc.* **2004**, *58*, 09-113.
64. Anyanwu, E. C.; Ehiri, J. E.; Kanu, I.; Morad, M.; Ventegodt, S.; Merrick, J. *ScientificWorld J.* **2004**, *4*, 978-988.
65. Bartlett, D. W.; Clough, J. M.; Godwin, J. R.; Hall, A. A.; Hamer, M.; Parr-Dobrzanski, B. *Pest Manag. Sci.* **2002**, *58*, 649-662.
66. Institut National de la Recherche Agronomique, INRA. *Phytoma* **2004**, *571*, 16-18.
67. De Jonghe, K.; De Dobbelaere, I.; Sarrazyn, R.; Hoefte, M. *Plant Pathol.* **2005**, *54*, 219-226.
68. Kim, B.S. 2001, KR Patent 2001036515, 2001.
69. Stanghellini, M. E.; Miller, R. M.; Rasmussen, S. L.; Kim, D. H.; Zhang, Y. WO Patent 9725866, 1997.
70. Environmental Protection Agency, Federal Register **2004**, *69*, 24984-24986.
71. Pavlik, M.; Vanova, M.; Laudova, V.; Harmatha, J. *Rostlinna Vyroba* **2002**, *48*, 543-547.
72. Shi, Z.; Shen, S.; Xu, L.; Fan, Y. *Nongyaoxue Xuebao* **2004**, *6*, 28-32.
73. Deng, Y.; Nicholson, R. A. *Planta Medica* **2005**, *71*, 364-365.
74. Del Rio, J. A.; Gomez, P.; Baidez, A. G.; Arcas, M. C.; Botia, J. M.; Ortuno, A. *J. Agric. Food Chem.* **2004**, *52*, 1913-1917.
75. Blount, J. W.; Dixon, R. A.; Paiva, N. L. *Physiol. Molec. Plant Pathol.* **1992**, *41*, 333-349.
76. Reyes-Chilpa, R.; Gomez-Garibay, F.; Moreno-Torres, G.; Jimenez-Estrada, M.; Quiroz-Vasquez, R.I. *Holzforschung* **1998**, *52*, 459-462.
77. Stevenson, P. C.; Turner, H. C.; Haware, M. P. *Physiol. Mol. Plant Pathol.* **1997**, *50*, 167-178.
78. Joshi, R. C.; Meepagala, K. M.; Sturtz, G.; Cagauan, A.G.; Mendoza, C. O.; Dayan, F. E.; Duke, S.O. *Int. J. Pest. Manag.* **2005**, *in press.*
79. Huang, H.-C.; Liao, S.-C.; Chang, F.-R., Kuo, Y.-H.; Wu, Y.-C. *J. Agric. Food Chem.* **2003**, *51*, 4916-4919.

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch001

80. Blodgett, D.; Ehrich, M.; Bloomquist, J. R. In *Handbook of Neurotoxicology*; Massaro, E. J, Ed.; Humana Press, Inc.: Totowa, NJ, 2002; pp 91-103.
81. Balbaa, S. I.; Khafagy, S. M.; Khayyal, S. E.; Girgis, A. N. *J. Nat. Prod.* **1979**, *42*, 522-524.
82. Chitwood, D. J. *Amer. Chem. Soc. Symp. Ser.* **1993**, *524*, 300-315.
83. Schrader, K. K.; De Regt, M. Q., Tidwell, P. R.; Tucker, C. S.; Duke, S. O. *Bull. Environ. Contam. Toxicol.* **1998**, *60*, 651-658.
84. Schrader, K. K.; Nanayakkar, N. P. D.; Tucker, C. S.; Rimando, A. M.; Ganzera, M.; Schaneberg, B. T. *Appl. Environ. Microbiol.* **2003**, *69*, 5319-5327.
85. Walker, D. R.; Boerma, H. R.; All, J. N.; Parrott, W. A. *Amer. Chem. Soc. Symp. Ser.* **2002**, *829*, 38-51.
86. Rajasekaran, K.; Cary, J. W.; Jacks, T. J.; Cleveland, T. E. *Amer. Chem. Soc. Symp. Ser.* **2002**, *829*, 97-117.
87. Kramer, K. J. In *Genetically Modified Crops*; Liang, G. H.; Skinner, D. Z., Eds; Haworth Press: Binghamton, NY, 2004; pp 119-130.
88. Yevtushenko, D. P.; Romero, R. R.; Forward, B. S.; Hancock, R. E.; Kay, W. W.; Misra, S. *J. Exp. Bot.* **2005**, *56,* 1685-1695.
89. Van Herpen, T. W. J. M.; Schwab, W.; Jongsma, M. A.; Bouwmeester, H. A.; Aharoni, A. *NATO Sci. Ser., Ser. I: Life Behav. Sci.* **2004**, *359*, 35-46.
90. Duke, S. O.; Scheffler, B. E.; Dayan, F. E.; Ota, E. *Weed Technol.* **2001**, *15*, 826-834.
91. Duke, S.O. *Trends Biotechnol.* **2003**, *21*, 192-195.

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch001

Downloaded by YORK UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch002

Chapter 2

Isolation of Seed Germination and Plant Growth Inhibitors from Mediterranean Plants: Their Potential Use as Herbicides

Marina DellaGreca[1,*], Francesca Cutillo[1], Brigida D'Abrosca[2], Antonio Fiorentino[2], and Armando Zarrelli[1]

[1]Department of Organic Chemistry and Biochemistry, University of Federico II, Monte S. Angelo, Via Cinthia 4, 1–80126 Naples, Italy
[2]Department of Life Science, Second University of Naples, Via Vivaldi 43, 81100 Caserta, Italy

The investigation of the extracts of plants *Cestrum parqui*, *Brassica fruticulosa*, and *Chenopodium album* led to the isolation of compounds belonging to different classes: C_{13} *nor*-isoprenoids, lignans and cinnamic acid amides. These compounds exhibit interesting activity on standard target species *Lactuca sativa*, *Lycopersicon esculentum*, and *Allium cepa* influencing seed germination and plant growth. Lignans are the most active products inhibiting lettuce germination, and the results show greater phytotoxic activity of these compounds with respect to commercial herbicides.

Introduction

Secondary metabolites isolated from higher plants could be useful natural herbicides (*1*, *2*). As part of our research on bioactive natural products isolated from mediterranean plants and their use as natural herbicide models, we report that some metabolites isolated from *Cestrum parqui*, *Chenopodium album*, and

In Natural Products for Pest Management; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

Brassica fruticulosa inhibit the germination and growth of some monocotyledonous and dicotyledonous plants. To evaluate the inhibitory or stimulatory effects on germination and seedling growth of plant extracts and pure substances, we selected as Standard Target Species two dicotyledonous plants, *Lactuca sativa* (lettuce) and *Lycopersicon esculentum* (tomato) and a monocotyledonous specie *Allium cepa* (onion). The assays were run in accordance with the procedures optimized by Macias groups (*3*).

Cestrum parqui

Cestrum parqui L'Herrit. 1788 (Solanaceae), commonly named green Cestrum, is a perennial shrub indigenous to South America, naturalised and now widely distributed in the Mediterranean area as one of the major weeds. It grows in dense masses, crowding out other species and it is noted for its extreme toxicity to farm animals (*4*). Pearce et al. (*5*) discovered the presence of *ent*-kaurene glycosides from the alcoholic extract of *C. parqui.*

Downloaded by YORK UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch002

Isolation and Structure Elucidation of C_{13} *nor*-Isoprenoids

Leaves of *C. parqui* were frozen at –80 °C, powdered and infused with H_2O containing 10% MeOH for 48 h. The extract was filtered with Whatman No.1 filter paper; the alcohol was removed *in vacuum* and the fraction was returned to the initial volume with deionised water. The crude extract and the 1:1 and 1:3 dilutions were tested on the selected species for the phytotoxic activity and the results are reported in Figure 1 as percentage of germination index (GI), calculated as follows:

$$\mathrm{GI}\,(\%) = \frac{\mathrm{RSG} \times \mathrm{RRG}}{100}$$

where RSG is the percentage of relative seed germination after 5 days for lettuce and tomato and 7 days for onion, and RRG is the relative root growth (*6*).

The aqueous extract showed a strong activity on the development of the three test species. The highest concentration showed about the 20% of germination index for the three test species. All the extracts showed a correspondence between concentration and effect. The aqueous fraction was extracted with methylene chloride, and chromatographic separation (silica gel CC, TLC and reverse phase HPLC) of the organic extract afforded the C_{13} *nor*-isoprenoids **1-12** (Figure 2) (*7*).

Downloaded by YORK UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch002

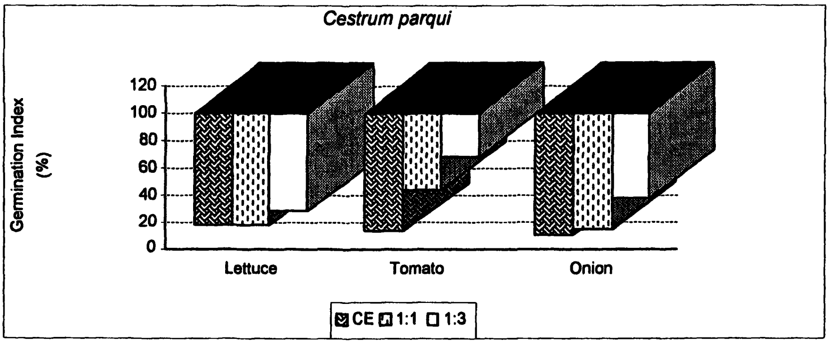


Figure 1. GI of the crude extract of C. parqui *on seeds of lettuce, tomato and onion. CE = crude extract; 1:1 = 1:1 dilution; 1:3 = 1:3 dilution.*

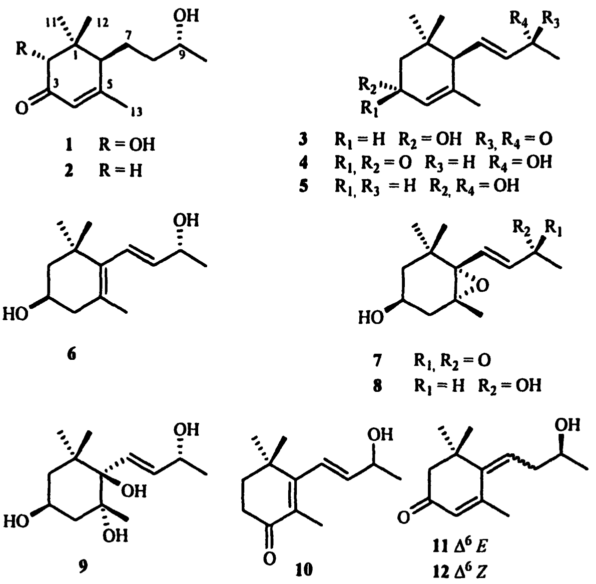

11
12
7
9
1
3
5
13
R
OH
O
1 R=OH
2 R=H
R4 R3
R2
R1
3 R1=H R2=OH R3,R4=O
4 R1,R2=O R3=H R4=OH
5 R1,R3=H R2,R4=OH
OH
HO
6
R2 R1
O
HO
7 R1,R2=O
8 R1=H R2=OH
OH
OH
HO
OH
9
OH
O
10
OH
O
11 Δ6 E
12 Δ6 Z

Figure 2. Structures of C_{13} nor-isoprenoids from C. parqui.

All the compounds were identified on the basis of their spectroscopic data (mass spectra, ^1H and ^{13}C NMR one and two-dimensional methods) and chemical correlation. The absolute configuration at carbinol carbons (C-3 and/or C-9) have been established by Mosher's method (*8*) for the **1-6**, **8**, **11** and **12** compounds. To define the configuration at C-2, of the (2*R*,6*R*,9*R*) 2,9-dihydroxy-4-megastigmen-3-one (**1**), an extension of Mosher method was utilised (*9*). The configuration at C-6 for the compounds **3-5** was derived by the CD spectrum that showed a positive Cotton effect at λ 230-250 nm (*10*). Compounds **11** and **12** were identified as geometric isomers at the Δ^6 double bond. These compounds were previously obtained by chemical synthesis, but this is the first time that they are reported as natural products.

The *R* configuration at C-6 of compound **2** was established by oxidation with MnO_2 to the known corresponding diketone, which had the same optical rotation. The structure of epoxide **7** was confirmed by semisynthesis starting from **6**, oxidation with MnO_2 gave the ketone at C-9, and subsequent treatment with peracid gave a mixture of the two epoxide with a large excess of α isomer. Mild acid hydrolysis of compound **8** gave as main product **9**.

In order to evaluate their potential phytotoxicity, all the compounds were tested on the seeds of *L. sativa*, with exception of **10** because of insufficient sample. The results are reported in Figure 3 for the most active compounds **1**, **3**, **4**, **7**, **9**, **11**, and **12**. Aqueous solutions of *nor*-isoprenoids, ranging between 10^{-4} and 10^{-7} M, were tested on the lettuce to evaluate their germination index. With the exception of the **3**, the compounds caused a germination index higher than 70%.

Downloaded by YORK UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch002

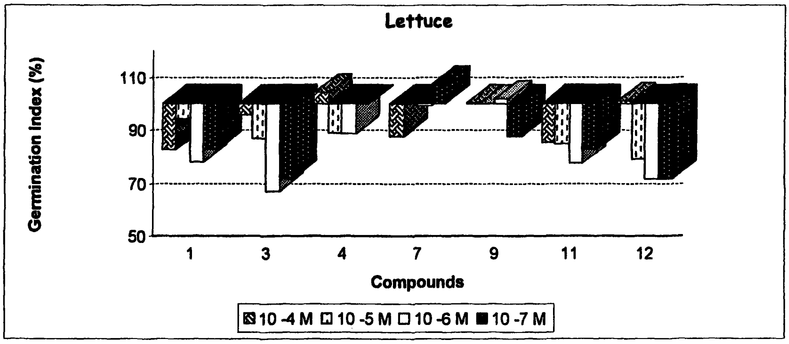


Figure 3. GI of C_{13} nor-isoprenoids from C. parqui *on lettuce seeds.*

Chenopodium album

Chenopodium album is an odorless, branching, largely annual weed diffused in cultivated fields (*11*), commonly known as lambsquarters. Mallik et al. (*12*) reported the presence of growth inhibitory substances in this plant. They found that the aqueous extract inhibited the germination and growth of radish and wheat seeds, attributing the activity to the presence of phenols. Horio et al. (*13*) reported the isolation of one phenolic amide with attracting activity toward the zoospores of *Aphanomyces cochlioides*.

Isolation and Structure Elucidation of Cinnamic Acid Amide

Crude aqueous fraction of *C. album* reduced the germination index of lettuce, tomato, and onion seeds to 80% of inhibition (Figure 4). The fraction was extracted with ethyl acetate and the organic layer was separated by conventional procedures into an acidic and a neutral fraction. The neutral portion was fractionated by silica gel column chromatography, and the fractions were purified by preparative layer chromatography and HPLC, yielding seven cinnamic amides **13-19** (Figure 5) (*14*).

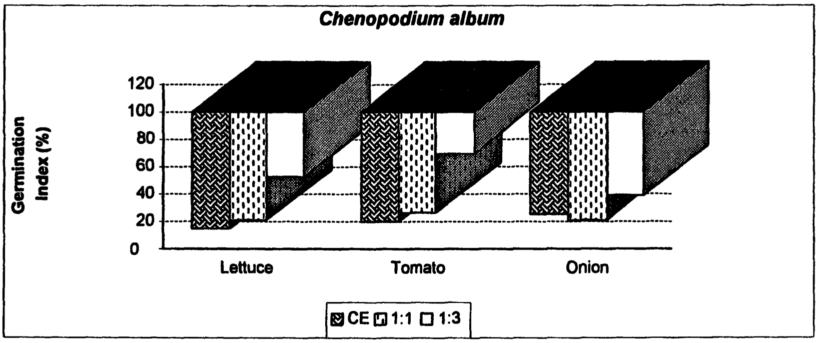
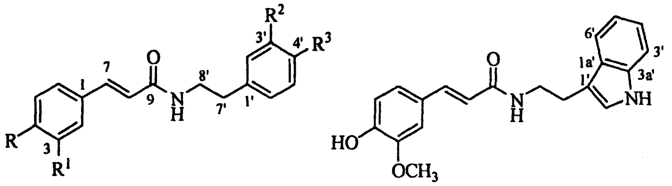


Figure 4. GI of the crude extract of C. album *on seeds of lettuce, tomato and onion. CE = crude extract; 1:1 = 1:1 dilution; 1:3 = 1:3 dilution.*

Compound **13** identified as *N-trans*-feruloyl 4'-*O*-methyldopamine, has been isolated from the roots of the same plant (*13*). Compounds **14-16** were already known: *N-trans*-feruloyl 3'-*O*-methyldopamine (**14**), *N-trans*-feruloyl tyramine (**15**) and *N-trans*-4-*O*-methylferuloyl 3',4'-*O*-dimethyldopamine (**16**) have been isolated from *Spinacia oleracea* (*15*), from *Hypecoum* sp. (*16*) and from *Zanthoxylum rubescens* (*17*), respectively. *N-trans*-4-*O*-methylcaffeoyl 3'-*O*-methyldopamine (**17**) and *N-trans*-feruloyl tryptamine (**18**) were reported only as synthetic substances (*18*). Compound **19** identified as *N-trans*-4-*O*-methylferuloyl 4'-*O*-methyldopamine was isolated for the first time from *C. album*.

All the cinnamic acid amides were tested on lettuce, tomato, and onion (Figure 6) to evaluate their germination index. All of the compounds showed a germination index higher of 70% on the dicotyledons with the exception of compound **15** which had phytotoxicity of about 50% on lettuce and about 40% on tomato. Stimulatory effects were observed on the onion for all the tested chemicals with the exception of compound **17** at the higher concentrations.

Downloaded by YORK UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch002



13 $R = R^2 = OH$ $R^1 = R^3 = OCH_3$
14 $R = R^3 = OH$ $R^1 = R^2 = OCH_3$
15 $R = R^3 = OH$ $R^1 = OCH_3$ $R^2 = H$
16 $R = R^1 = R^2 = R^3 = OCH_3$
17 $R = R^2 = OCH_3$ $R^1 = R^3 = OH$
19 $R = R^1 = R^3 = OCH_3$ $R^2 = OH$

18

Figure 5. Structures of cinnamic acid amides from C. album.

Brassica fruticulosa

Continuing the phytochemical study of common weeds widely distributed in the Mediterranean area, we have investigated *Brassica fruticulosa* Cyr., a plant belonging to the large family of Brassicaceae. The analyses of the extracts led to the isolation of lignans, neolignans, sesquilignans and one dilignan. Lignans are derived from the phenylpropanoid pathway and are widely distributed in plants.

Isolation and Identification of Lignans and Neolignans

The water/methanol extract of *B. fruticulosa* was partitioned between methylene chloride and water. The organic extract was fractionated by silica gel column chromatography and the fractions were purified by preparative TLC, DCCC and HPLC, yielding four lignans, three neolignans, one sesquilignan, and one dilignan. The aqueous fraction was chromatographed on Amberlite XAD-2 and fractionated by Sephadex LH-20 column chromatography. The fractions containing aromatic compounds were purified by preparative TLC, DCCC and HPLC, yielding one lignan, two neolignans, and one sesquilignan (Figures 7-9) (*19*).

The lignans were identified by comparison with previously reported spectroscopic data (*20-23*). These compounds were (-)-pinoresinol (**20**), (-)-isolariciresinol (**21**), (+)-secoisolariciresinol (**22**), (±)-lariciresinol (**23**) and (-)-tanegol (**24**). A comparison of the reported optical rotations for the pure enantiomers with the value measured indicated an enantiomeric excess of 30% for compound **20**, 20% for compound **21**, and 6% for compound **24**. The neolignan **25** was dehydrodiconiferyl alcohol isolated as a racemic mixture. The

Downloaded by YORK UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch002

Downloaded by YORK UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch002

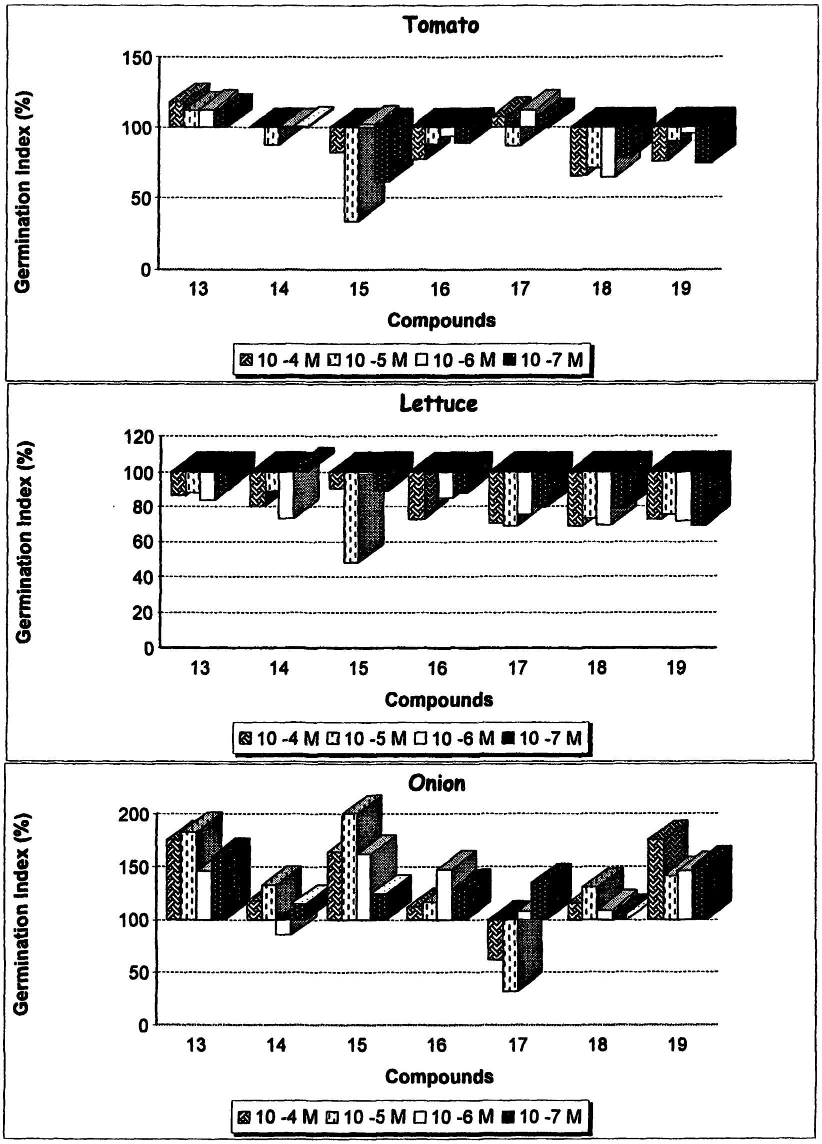


Figure 6. GI of cinnamic acid amides from C. album *on seeds of lettuce, tomato, and onion.*

Downloaded by YORK UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch002

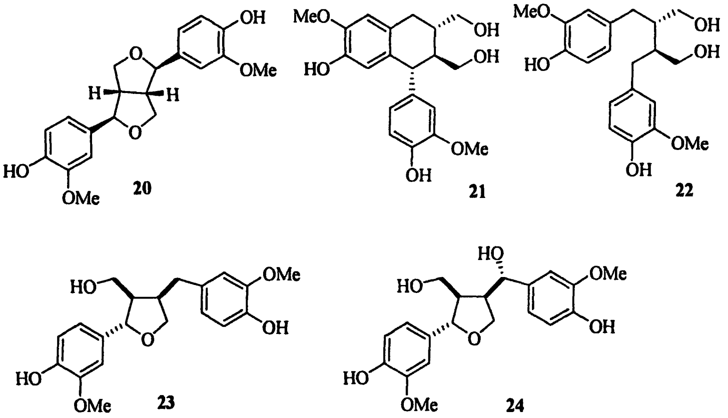


Figure 7. Structures of lignans from B. fruticulosa.

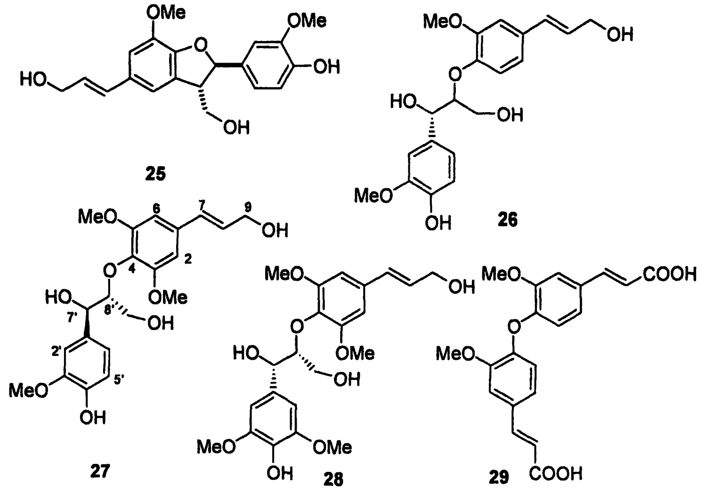


Figure 8. Structures of neolignans from B. fruticulosa.

other neolignans were (±)-*threo*-guaiacylglycerol-β-*O*-4'-coniferyl ether (**26**), (+)-*threo*-guaiacylglycerol-β-*O*-4'-synapyl ether (**27**), (±)-*erythro*-syringylglycerol-β-*O*-4'-sinapyl ether (**28**). The *threo* relative configuration for compounds **26** and **27** was predicted on the basis of the $^3JH_7H_8$ NMR coupling constant value reported in the literature (*24*). The absolute configuration 7*R*, 8*R* for compound **27** derived from the negative CD curve in the 210-250 nm range

(*25*). This compound was isolated as a glycoside from the bark of *Eucommia ulmoides* (*26*). The EIMS spectrum of compound **28** had a molecular peak at *m/z* 436 consistent with a molecular formula $C_{22}H_{28}O_9$. It showed only 17 carbon signals in the ^{13}C NMR spectrum and a DEPT experiment defined the carbons as two methyls, two methylenes, and seven methines. In accordance with the *erythro* relative configuration, a coupling constant value of 4.0 Hz between H-7 and H-8 was observed. Compound **29** was identified as 1-feruloyloxy-2-methoxycinnamic acid and was identified by comparison with previously reported spectroscopic data (*27*).

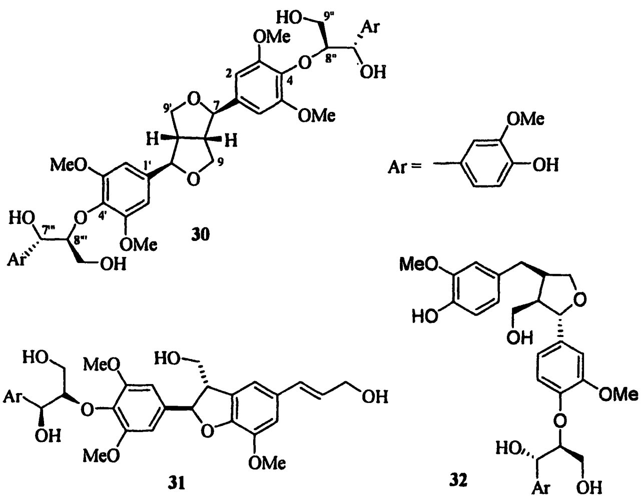


Figure 9. Structures of dilignan and sesquilignans from B. fruticulosa.

Compound **30** was identified as a dilignan. In the ^{13}C NMR spectrum only twenty-one carbon signals were present indicating a highly symmetric molecule. In the MALDI/MS the molecular ion at *m/z* 833 $[M+Na]^+$ was present. The ^1H NMR and COSY spectra revealed the connectivities of four protons characteristic of the 3,7-dioxabicyclo [3.3.0] octane and propane 1,2,3-triol groups. The above data matched those reported by Matsuda et al. (*28*) for hedyotisol-A isolated from *Hedyotis lawsoniae* and identified as a hexaacetate. Compound **31** had molecular formula $C_{31}H_{36}O_{11}$ according to the molecular ion at *m/z* 607 $[M+Na]^+$ in the MALDI/MS spectrum and elemental analysis. The ^{13}C and ^1H NMR spectra showed the presence of one aromatic ring with three coupled protons in a ABX system, and two aromatic rings, each one with two protons, which were located at *meta* sites relative to each other. The presence of a *trans* double bond was confirmed by ^1H NMR spectrum. The COSY spectrum

Downloaded by YORK UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch002

Downloaded by YORK UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch002

revelead a glycerol moiety like C-7"–C-9" and a neolignan with benzofuran ring C-7'–C-9'. In accordance with to the *erythro* relative configuration the coupling constant value of 4.5 Hz between H-7" and H-8" was observed. These data resembled that of the aglycone of alangisesquin A isolated from ***Alangium premnifolium*** (*29*) and buddlenol B isolated from ***Buddleja davidii*** (*30*).

Compound **32** revealed a $[M+Na]^+$ peak at *m/z* 579 in the MALDI/MS spectrum, suggesting the molecular formula, $C_{30}H_{36}O_{10}$ confirmed by elemental analysis. The ^{13}C NMR spectrum of **32** showed 27 carbon signals except for the three methoxyl signals, indicating **32** to be a sesquilignan. The ^1H and ^1H-^1H COSY spectra showed the presence of three sets of ABX patterns in the aromatic region and a glycerol and a tetrahydrofuran. The DEPT experiment defined the carbons as three methyls, four methylenes, fourteen methines and nine quaternary carbons. The HMQC experiment allowed assigning the protons to the corresponding carbons. The connection of functional groups was determined on the basis of HMBC correlations. Careful analysis of fragmentations of the molecular ion in the MALDI/MS, showed peaks at *m/z* 561 $[M+Na-H_2O]^+$ (18%), 549 $[M+Na-CH_2O]^+$ (10), 531 $[M+Na-H_2O-CH_2O]^+$ (37) and 382 $[M+Na\text{-}guaiacylglicerol]^+$ (100). The significant relative abundances of peaks at *m/z* 412 $[M+Na-CH_2O-C_8H_9O_2]^+$ and 342 $[M+Na-C_{13}H_{17}O_4]^+$ indicated the presence of guaiacylglicerol unit at *O*-4'.

All the lignans isolated from *B. fruticulosa* were tested on the lettuce, and onion, except compound **28**, isolated in small quantities. The compounds **20-21**, **23-27** and **29** were also assayed on the tomato (*19*). The GI for the most active compounds are reported in Figures 10-12. The most relevant effect observed was a strong inhibition of *L. sativa* produced by compounds **23**, **25** and **26**, (Figure 10). Lignan **23** is the most active with GI 21% at the lower concentration (10^{-9} M). Among the neolignans, dehydrodiconiferyl alcohol (**25**) showed about 70% of inhibition at all the tested concentrations. The dilignan **30** and the sesquilignans **31** and **32** were phytotoxic on the lettuce, too. The effects on the germination index of *L. esculentum* and *A. cepa* were not substantial (Figures 11 and 12).

The high values of inhibiting activity due to compounds **23**, **25-26** and **30-32**, suggested their potential use as natural pesticides. Therefore, these six natural compounds were compared with pendimethalin, a commercial pre-emergence herbicide widely used in agriculture (*31*). The results, reported in Figure 13, showed the major phytotoxic activity on lettuce germination of compounds **23**, **25** and **26** with respect to the herbicide from 10^{-7} to 10^{-9} M concentrations. Pendimethalin acts as an inhibitor of cell division and elongation (*32*).

Downloaded by YORK UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch002

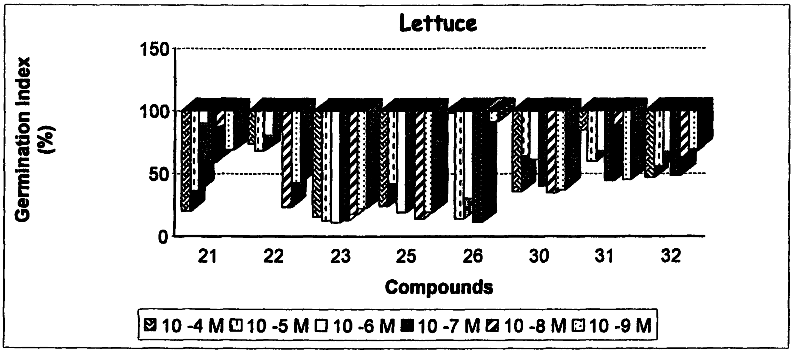


Figure 10. GI of compounds 21-23, 25-26, and 30-32 from B. fruticulosa *on lettuce.*

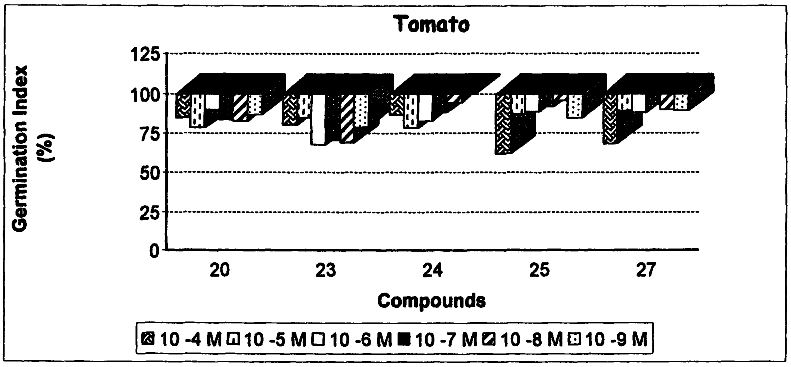


Figure 11. GI of compounds 20, 23-25, and 27 from B. fruticulosa *on tomato.*

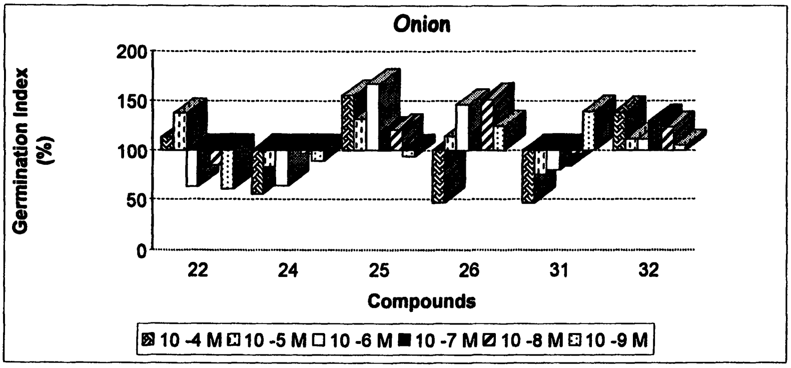


Figure 12. GI of compounds 22, 24-26, and 31-32 from B. fruticulosa *on onion.*

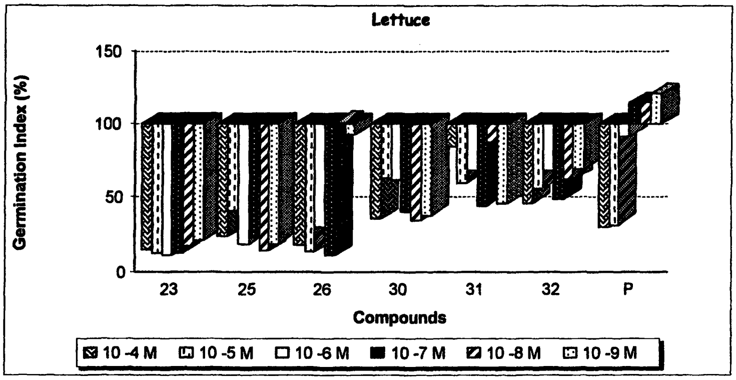


Figure 13. Comparison of the bioactive lignans 23, 25-26, and 30-32 with pendimethalin (P).

The results of the phytotoxic activity of the lignans from *B. fruticulosa* confirmed their potential phytotoxic role and the relative natural abundance of these metabolites suggested their potential use as selective natural pesticides.

References

1. Lin, L. J.; Peiser, B. P.; Ying, K.; Mathias, F.; Karasina, Z.; Wang, Z.; Itatani, J.; Green, L.; Yang, S. *J. Agric. Food Chem.* **1995,** *43*, 1708-1711.
2. Heisey, R. *Amer. J. Bot.* **1990,** *77*, 662-670.
3. Macias, F. A.; Castellano, D.; Molinillo, J. M. G. *J. Agric. Food Chem.* **2000,** *48*, 2512-2521.
4. McLennan, M. W.; Kelly, W. R. *Aust. Vet. J.* **1984,** *61*, 289-291.
5. Pearce, C. M.; Skelton, N. J.; Naylor, S.; Kanaan, R.; Kelland, J.; Oelrichs, P. B.; Sanders, J. K. M.; Williams, D. H. *J. Chem. Soc. Perkin Trans 1* **1992,** 593-600.
6. Hoekstra, N. J.; Bosker, T.; Lantinga, E. A. *Agr. Ecosyst. Environ.* **2002,** *93*, 189-196.
7. D'Abrosca, B.; DellaGreca, M.; Fiorentino, A.; Monaco, P.; Oriano, P; Temussi, F. *Phytochemistry* **2004,** *65*, 497-505.
8. Dale, J. A.; Mosher, H. S. *J. Am. Chem. Soc.* **1973,** *95*, 512-519.
9. Ohtani, I.; Kusumi, T.; Kashman, Y.; Kakisawa, H. *J. Am. Chem. Soc.* **1991,** *113*, 4092-4096.
10. Harada, N.; Nakanishi, K. *Circular Dichroic Spectroscopy*; University Science Books, Oxford University Press: Oxford, 1983; p 240.

Downloaded by YORK UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch002

11. Holm, L.; Pluncknett, D.; Pancho, J.; Herberger, A. *World's worst Weeds: Distribution and Biology*. University of Hawaii Press: Honolulu, 1977; p 209.
12. Mallik, M. A. B.; Puchala, R.; Grosz, F. A. *J. Chem. Ecol.* **1994,** *20*, 957-967.
13. Horio, T.; Yoshida, K.; Kikuki, H.; Kawabata, J.; Mizutani, J. *Phytochemistry* **1993,** *33*, 807-808.
14. Cutillo, F.; D'Abrosca, B.; DellaGreca, M.; Di Marino, C.; Golino, A.; Previtera, L.; Zarrelli, A. *Phytochemistry* **2003,** *64*, 3307-3330.
15. Suzuki, T.; Holden, I.; Casida, J. *J. Agric. Food Chem.* **1981,** *29*, 992-995.
16. Hussain, S. F.; Gözler, B.; Shamma, M.; Gözler, T. *Phytochemistry* **1982,** *21*, 2979-2980.
17. Adesina, S. K.; Reish, J. *Phytochemistry* **1989,** *28*, 839-842.
18. Suzuki, T.; Holden, I.; Casida, J. *J. Agric. Food Chem.* **1981,** *29*, 992-995.
19. Cutillo, F;. D'Abrosca, B.; DellaGreca, M.; Fiorentino A.; Zarrelli, A. *J. Agric. Food Chem.* **2003,** *51*, 6165-6172.
20. Lin-gen, Z.; Seligmann, O.; Jurcic, K. K.; Wagner, H. *Planta Med.* **1982,** *45*, 172-176.
21. Urones, J. G.; De Pascual, Teresa, J.; Sanchez Marcos, I.; Diez Martin, D. *Phytochemistry* **1987,** *26*, 1540-1541.
22. Fonseca, S. B.; De Paiva Campello, J.; Barata, L. E. S.; Ruveda, E. A. *Phytochemistry* **1978,** *17*, 499-502.
23. Abe, F.; Yamauchi, T. *Chem. Pharm. Bull.* **1990,** *38*, 2143-2145.
24. Besombes, S.; Robert, D.; Utille, J.; Taravel, F. R.; Mazeau, K. *J. Agric. Food Chem.* **2003,** *51*, 34-42.
25. Della Greca, M.; Molinaro, A.; Monaco, P.; Previtera, L. *Phytochemistry* **1994,** *35*, 777-779.
26. Takeshi, D.; Takako, I.; Shizuka, K.; Sansei, N. *Chem. Pharm. Bull.* **1987,** *35*, 1803-1807.
27. Huang, J.; Ogihara, Y.; Gonda, R.; Takeda, T. *Chem. Pharm. Bull.* **2000,** *48*, 1228-1229.
28. Matsuda, S.; Kadota, S.; Tai, T. *Chem. Pharm. Bull.* **1984,** *32*, 5066-5069.
29. Kuima, K.; Otzuka, H.; Ide, T.; Ogimi, C.; Hirata, E.; Takushi, A.; Takeda, Y. *Phytochemistry* **1998,** *48*, 669-676.
30. Houghton, P. J. *Phytochemistry* **1985,** *24*, 819-826.
31. Strandberg, M.; Scott-Fordsmand, J. J. *Ecotox. Environ. Safety* **2004,** *57*, 190-201.
32. Richard, G. A.; Hussey, P. J. *Trends Plant Sci.* **1999,** *4*, 112-116.

Downloaded by YORK UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch002

Chapter 3

Chlorosis Inducing Phytotoxic Metabolites: New Herbicides from *Phoma macrostoma*

Paul R. Graupner[1], B. Clifford Gerwick[1], Thomas L. Siddall[1], Andy W. Carr[1,3], Erin Clancy[1], Jeffrey R. Gilbert[1], Karen L. Bailey[2], and Jo-Ann Derby[2]

[1]Dow AgroSciences, 9330 Zionsville Road, Indianapolis, IN 46268
[2]Agriculture and Agri-Food Canada, 107 Science Place,
Saskatoon S7H OX2, Canada
[3]Current address: Eli Lilly and Company, Lilly Corporate Center,
Indianapolis, IN 46285

Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch003

In the course of screening for new phytotoxic metabolites that produce severe chlorosis, we identified a new family of herbicides, the macrocidins. Field isolates of *Phoma macrostoma* were obtained from chlorotic and diseased Canada thistle and were grown in liquid culture. These were found to produce phytotoxic metabolites which also caused bleaching and chlorosis when applied as extracts to several broadleaf species. Bioassay directed isolation led to identification of two major factors, macrocidin A and B. These are the first representatives of a new family of cyclic tetramic acids. These compounds were also produced synthetically, using olefin metathesis to close the macrocycle. In addition, some cyclic and non-cyclic analogs were also prepared in order to probe the structure/activity relationship.

In Natural Products for Pest Management; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch003

A number of chlorosis inducing metabolites have been described from fungi, bacteria and higher plants, including phosphonothrixin (*1*), leptospermone (*2*), adenyl oxetane (*3*), and tentoxin (*4*). Where known, these natural products affect different target sites, and the bleaching and loss of photosynthetic pigments in treated plants can result from a number of direct and indirect means. Among commercial bleaching herbicides, the inhibition of hydroxyphenyl pyruvate dioxygenase (HPPD), originally described for leptospermone, is of particular significance. A wide range of chemistries from natural and synthetic sources have subsequently been identified as inhibitors of this target, which has resulted in a broad range of pre and post-emergence herbicides with a number of unique attributes and significant grower value. Given the significance of bleaching herbicides to commercial weed control, target sites resulting in this phenotype have been suggested for further investigation and potential future herbicide discovery (*5*).

We have been particularly interested in bleaching herbicides that are phloem mobile and have identified a number of new compounds from natural and synthetic sources with this property. During the course of testing extracts of microbial origin for herbicide activity, we identified potent bleaching activity in extracts from *Phoma macrostoma* (*6*). In this report we present the discovery of a new family of bleaching natural products, the macrocidins (Figure 1), from this fungal pathogen, and the synthesis and testing of close structural analogs.

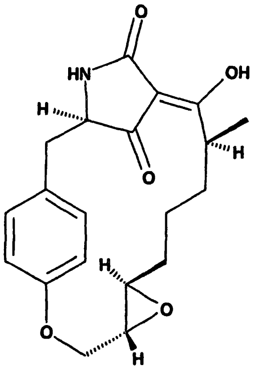


Figure 1: Macrocidin A (1)

Finding novel natural products typically requires the screening of large numbers of biologically-derived extracts. However, observation of symptoms on diseased plants in the field can also be an effective method to discover novel compounds. *Phoma macrostoma* was isolated from diseased thistle plants showing severe chlorotic symptoms. Releasing the fungus into soil resulted in severe bleaching symptoms on emerging plants in the greenhouse. Further testing revealed that the active components could be extracted from the fungal mycelia into chloroform, and applied to the plants as a methanolic solution. Additionally, extracts applied directly to the soil affected the growth of newly germinating plants, in a manner similar to those caused by a direct application of the fungus to soil, with symptoms of severe bleaching and growth retardation with phytotoxic effects reaching down to the roots.

Phoma macrostoma is a filamentous fungus which has been associated with plant disease, for example causing black rot of artichoke and also leaf-spot disease of Indian jujube plants. Despite this known phytopathogenicity, no herbicidal compounds have been reported from this particular fungus. However, phytotoxic compounds have been reported from other *Phoma* species; for example Amphidicolin from *Phoma betae* which inhibits root growth in lettuce seedlings (*7*), and Phomalirazine, a diketopiperazine from *Phoma lingam*, the causal agent for black leg in canola (*8*) (Figure 2).

Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch003

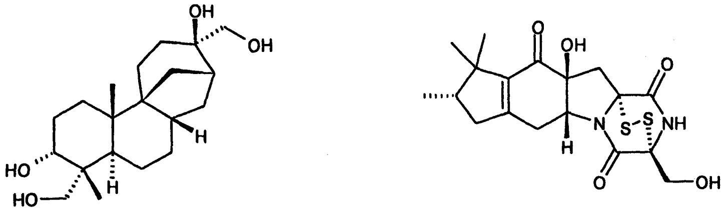


Figure 2: Known phytotoxic compounds from Phoma species: from left, amphidicolin, and phomalirazine.

Bioassay directed isolation of *P. macrostoma* extracts revealed a cluster of compounds with the same unusual UV spectra with maxima at 226 and 280nm. The most abundant molecule was determined to be the unusual macrocyclic tetramic acid macrocidin A (**1**) the structure determination of which has been previously described (*6*). Chromatographic analysis, however, revealed a number of different molecules with the same UV response, but in much less abundance (Figure 3). Proton NMR revealed that these molecules differed from **1** by the absence of the epoxide (**2**) or by hydroxylation at various locations (for example **3-5**). These minor factors were only available in very small amounts which precluded acquisition of a full analytical data package corresponding to their absolute structures, and not allowing for full biological evaluation of their activity. However, the activity of the compounds was sufficiently interesting to warrant investigating the SAR for this novel class of chemistry and so a small synthetic program to investigate the chemistry of these molecules was initiated.

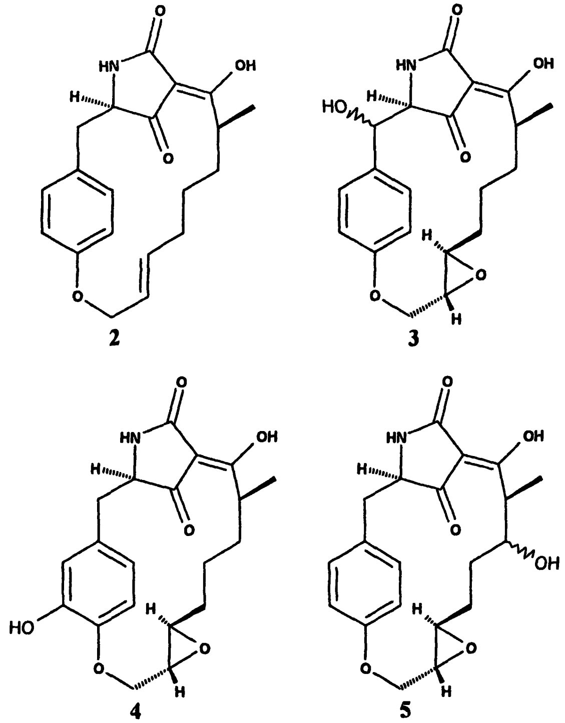


Figure 3: Structures of naturally occurring macrocidin derivatives; note that paucity of sample precluded determination of relative stereochemistry in the hydroxylated derivatives.

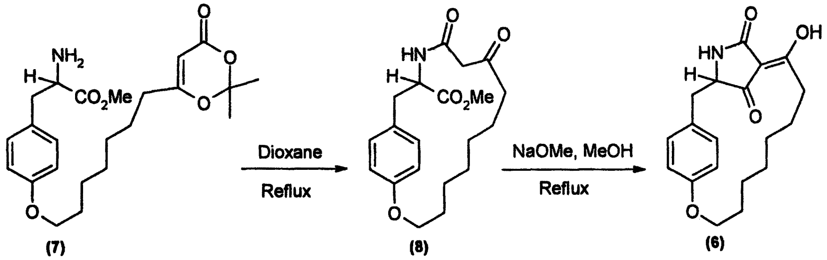
Synthesis of Macrocidins and Unnatural Analogs

In order to probe structural features necessary for herbicidal activity, the first targeted compound prepared in this series, **6**, contained a simple unsubstituted alkyl backbone linking the acyl tetramate and tyrosine moieties. This compound was prepared by a straightforward application of the macrolactamization / tetramic acid formation protocols reported in Boeckman's synthesis of (+)-ikarugamycin (*9*). The macrocyclization step is carried out by high dilution thermolysis of the acylketene precursor **7**, leading to the lactam **8**. Dieckmann cyclization of **8** with NaOMe in refluxing methanol produces the target tetramate **6**.

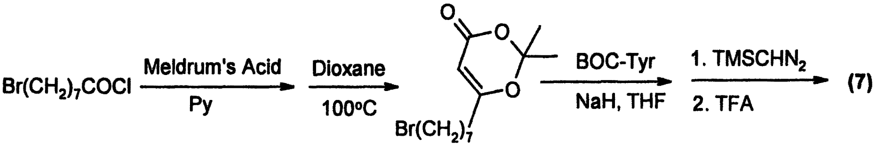
The tyrosine derivative **7** was assembled in four steps from 8-bromooctanoyl chloride.

Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch003

Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch003

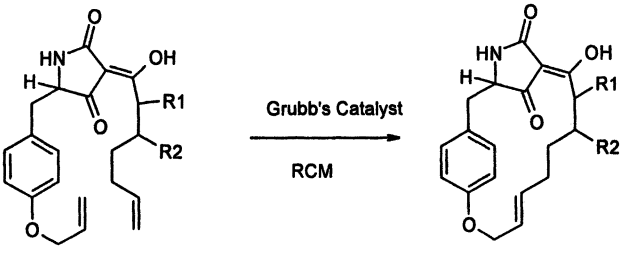

NH2
H
CO2Me
O
O
O
O
Dioxane
Reflux
HN
H
CO2Me
O
O
NaOMe, MeOH
Reflux
HN
H
OH
O
O
(7)
(8)
(6)

Scheme 1: Synthesis of macrocidin derivative forming the acyl tetramic acid as the last step.


Br(CH2)7COCl
Meldrum's Acid
Py
Dioxane
100°C
O
O
O
Br(CH2)7
BOC-Tyr
NaH, THF
1. TMSCHN2
2. TFA
(7)

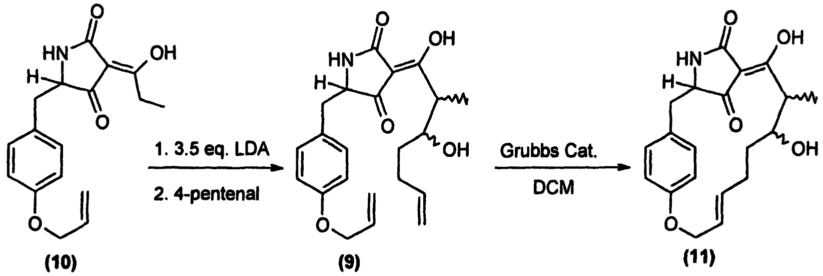
Scheme 2: Synthesis of pre-macrocyclization intermediate 7.

Preparation of the more substituted analogs would require specifically substituted 8-halooctanoates which can be difficult to access so a more efficient route was developed in which the macrocyclization of tetramate dienes was effected with ring-closing olefin metathesis (RCM) using the catalysts pioneered by Grubbs[10] (Scheme 3). This also, of course, produces the requisite olefin precursors to the epoxides present in many of the natural macrocidins.


HN
H
O
OH
R1
R2
O
O
Grubb's Catalyst
RCM
HN
H
O
OH
R1
R2
O
O

Scheme 3: Synthesis of substituted macrocidins using RCM as the final step.

The mild RCM conditions have the added advantage of being less likely to induce racemization at the tyrosine α-carbon than the somewhat forceful Dieckmann conditions required to form the tetramate post-macrocyclization. It was not necessary to protect the tetramic acid functionality during this process and the reactions were typically performed under only moderate dilution (10^{-2} molar). The unoptimized yields were moderate (15-60%) but acceptable considering the brevity of this route. All reactions were performed with Grubb's first generation catalyst. Yield enhancements might be realized using later generation catalysts. NMR analysis indicated > 95% selectivity to the (*E*)-olefin which upon epoxidation should lead to the same stereochemistry found in the macrocidin epoxides. The scope of this reaction has barely been explored, but seems to adequately tolerate acidic and hydroxylic groups that can be problematic in some RCM reactions. The RCM diene substrates were prepared starting with O-allyl tyrosine methyl ester and introducing the acyl olefin side-chain *via* standard tetramic acid chemistry using the appropriate olefinic acid chlorides as in Scheme 2. The α-methyl-β-hydroxy diene **9** was quickly assembled by application of tetramic acid trianion chemistry (*11*). Triple deprotonation of the propanoyl tetramate **10** with 3.5 eq. LDA followed by addition of 4-pentenal gives the hydroxydiene as a mixture of diastereomers (Scheme 4). T his mixture was subjected to RCM to give the diasteromeric macrocidins **11** that were separable by preparatory RP HPLC. Epoxidation of the macrocidin olefins is currently being studied.



Scheme 4: Synthesis of hydroxylated macrocidin derivatives.

Activity (SAR)

All molecules prepared using the above chemistry - both cyclic and acyclic - were screened for herbicidal activity against a number of target species. From these results, the following observations regarding the SAR were made:

- Broadleaf weeds were more sensitive than grass weeds to this chemistry
- The epoxide was not required for good activity; a double bond in the same position gave similar activity levels.
- Substituting saturated carbon atoms for the olefin was detrimental to activity.
- The methyl group next to the acyl tetramate was important for good activity, but not essential.
- Substitution of oxygen for one of the methylenes in the macrocycle eliminated activity

Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch003

- Addition of a hydroxy group at many positions (either on the tyrosine fragment or on the backbone) improved activity
- Some activity was noted for simple non-macrocyclized acyl tetramic acid derivatives [*c.f.* (structure **11**, Figure 5)]

Mode of action

With the observation that simple acyclic tetramic acids exhibited bleaching activity, attention was drawn to the mode-of-action of the macrocidins. As mentioned in the introduction, inhibition of HPPD is of particular significance. Although it was believed that the activity exhibited by the fungal extract was not consistent with HPPD inhibition (based on the macrocidins inhibiting root growth as well as causing foliar bleaching), it had already been shown that some simple acyl tetramic acid molecules did indeed inhibit this enzyme (data not shown). Therefore it was decided to compare the mode of action of some of the compounds.

The role of HPPD in the biosynthesis of tocopherols and plastoquinone is shown in Figure 4. Thus hydroxyphenylpyruvate, which is derived from tyrosine, is rearranged by HPPD to homogentisic acid. Reversal experiments using homogentisic acid can therefore be used to determine whether activity is due to inhibition of this pathway.

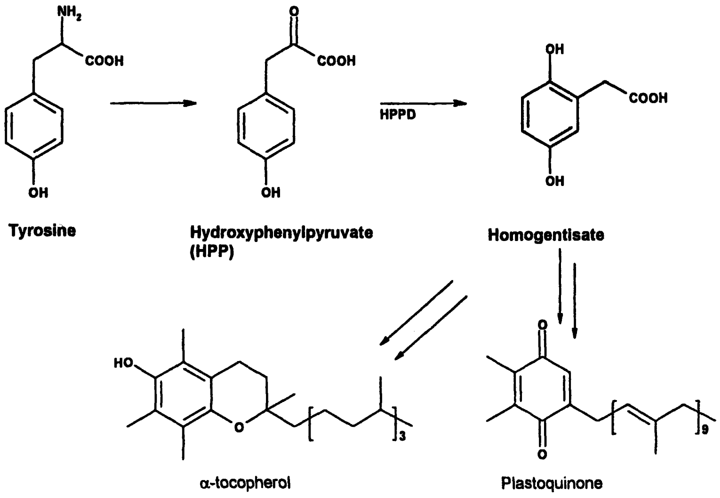
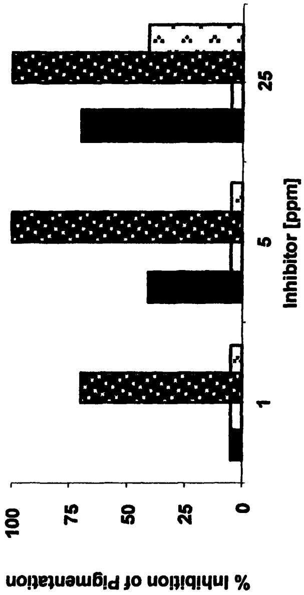
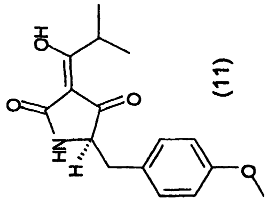


Figure 4: Role of HPPD in the biosynthetic pathway of tocopherols and plastoquinone from tyrosine. Plastoquinone is an essential cofactor for phytoene desaturase.

Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch003

Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch003





(11)

Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch003

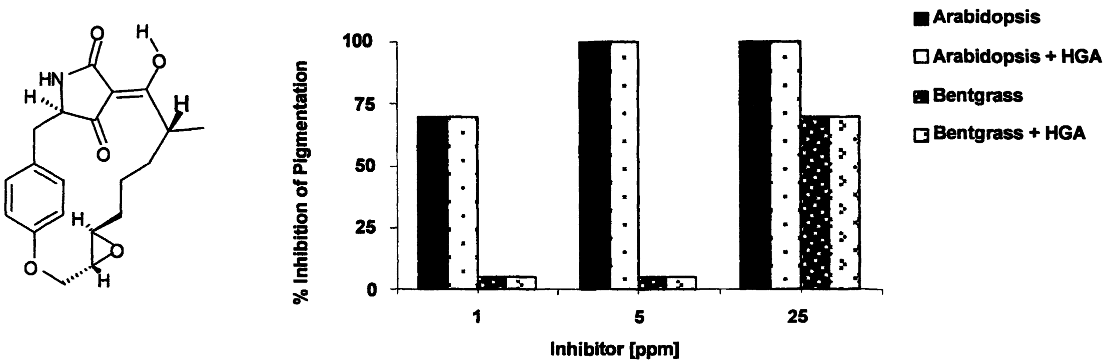


Figure 5: Results of reversal experiments using homogentisic acid. The upper graph illustrates complete activity reversal on Arabidopsis and bentgrass seedlings when homogentisic acid is added to the plates, indicating the uncyclised acyl tetramic acid derivative acts through the HPPD pathway. Macrocidin A however is still active in the presence of HGA, suggesting non-HPPD activity (lower).

Figure 5 shows the results of these experiments on two molecules from this series. Agar-based plate assays (*12*) using Arabidopsis and bentgrass clearly showed activity from both the acyclic tetramic acid derivative and the macrocidin derivative. In the case of the tyrosine acyl tetramic acid (**11**), the activity was completely reversed by addition of homogentisic acid. For macrocidin A, the activity was still apparent after this addition. These data suggest that the mode of action of the macrocyclic compounds is different than **11**, and may be worthy of further investigation. At present, the mode of action of the macrocidins is unknown.

Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch003

Conclusions

The macrocidins are a novel class of chemistry which cause growth retardation of shoot and root systems, and severe bleaching. Synthesis of the molecules is straightforward making the family a good target for herbicide discovery. Even though non-macrocyclic analogs demonstrated activity against the HPPD pathway, this does not appear to be the mode of action of the macrocidins. This mode of action is presently unknown and may lead to novel areas of chemistry for exploitation in the future.

References

1. Wakabayashi, K.; Boeger, P. *Weed Biology and Management* **2004,** *4,* 8.
2. Kimura, T.; Nakamura, K.; Takahashi, E. *J. Antibiotics* **1995,** *48,* 1130.
3. Lax, A. R.; Shepherd, H. S. ACS Symposium Series (1988), 380 (Biol. Act. Nat. Prod.: Potential Use Agric.), 24.
4. Lee, D. L.; Prisbylla, M. P.; Cromartie, T. H.; Dagarin, D. P.; Howard, S. W.; Provan, W. M.; Ellis, M. K.; Fraser, T.; Mutter, L. C. *Weed Science* **1997,** *45,* 601.
5. Gerwick, B. C.; Graupner, P. R.; Gray, J. A.; Peacock, C. L.; Hahn, D. R.; Chapin, E. L; Schmitzer, P. R. *International Weed Control Congress:* **2000,** Foz De Iguassu: Brazil.
6. Graupner, P. R.; Gerwick, B. C.; Carr, A.; Clancy, E.; Gilbert, J.; Bailey, K. L.; Derby, J-A. *J. Nat. Prod.* **2003,** *66,* 1558.
7. Sakai, R.; Mino, Y.; Ichihara, A.; Sakmura, S. *Nippon Shokubutsu Byori Gakkaiho* **1985,** *51,* 219.

8. Pedras, M. S. C.; Abrams, S. R.; Seguin-Swartz, G; Quail, J. W.; Jia, Z. *J. Amer. Chem. Soc.* **1989**, *111*, 1904.
9. Boeckman, R. K.; Weidner, C. H.; Perni, R. B.; Napier, J. J. *J. Amer. Chem. Soc.* **1989**, *111,* 8036-8037.
10. Miller S. J., Blackwell H. E.; Grubbs, R. H. *J. Amer. Chem. Soc.* **1996**, *118*, 9606.
11. Jones, R. C. F.; Bates, A. D. *Tetrahedron Letts.* **1987**, *28*, 1565.
12. Cseke, C.; Gerwick, B. C.; Crouse, G. D.; Murdoch, M. G.; Green, S. B.; Heim, D. R. *Pest. Biochem and Physiol.* **1996**, *55*, 210.

Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch003

Downloaded by PENNSYLVANIA STATE UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch004

Chapter 4

Physiological Effects of 2-Benzoxazolinone on Lettuce

Manuel J. Regiosa and Adela M. Sánchez-Moreiras

**Department of Plant Biology and Soil Science, University of Vigo,
As Lagoas, Marcosende, s/n, Vigo 36310, Spain**

The authors have proposed a general mode of action for 2-benzoxazolinone (BOA), including several primary effects and their relation to general stress pathways, including sencescence and oxidative damage. Experiments were performed using whole plants with the addition of BOA, BOA + NaCl and NaCl alone to elucidate the validity of this mode of action. Results partially support the proposed mode of action.

Primary and Secondary Effects of 2-Benzoxazolinone

Allelopathic properties of 2-benzoxazolinone (BOA) have been studied for the past few years in our laboratory (*1*). BOA has been shown to produce several primary and secondary effects in laboratory conditions: it slows root cell cycle (*1*), depresses germination (*2*), deorganizes cell membranes (*3*) and changes gene expression (*1*).

According to those physiological effects, a general multiple mode of action model has been proposed (*1*). In this work we try to validate the effects at the whole plant level using combination of abiotic (salt) and biotic stress, considering that the proposed model suggests that the effects of BOA are

In Natural Products for Pest Management; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

correlated partially to the stress response and that it is probable to have some kind of synergy between allelopathic and non-allelopathic stresses (*4*). Therefore, we selected the occurrence of salt stress by its common and increasing presence in limiting plant productivity in several agricultural lands (*5*).

Experimental designs

Lettuce seeds (*Lactuca sativa* cv. Great Lakes, California) were germinated in plastic trays with a 5-cm deep layer perlite and filled every other day with 500 mL 1:1 Hoagland's solution (*6*). Seedlings were germinated in a controlled environment chamber with 20 °C temperature (in dark). For seedling growth the environmental conditions were: 12 h-photoperiod, 18:8 °C day-night temperature, 60 ± 5% relative humidity, and 200 μmol m^{-2} s^{-1} irradiance. One month after germination, plants were transferred to pots containing perlite. One week later, roots were exposed to four different treatments for 15 days: control (i.e. Hoagland's solution 1:1), 1 mM BOA, 60 mM NaCl, and a combined treatment of 1 mM BOA + 60 mM NaCl.

Photosynthesis and transpiration, recorded with an infrared gas analyser LI-6400 (Li-Cor model 6400, Lincoln, USA), were measured on the first well-expanded leaf in six replicates per treatment and pulse-modulated and continuous fluorescence measurements, recorded with a Plant Efficiency Analyser (Hansatech Instruments Ltd.). Fresh and oven dry plant weight, relative water content (RWC), water potential (Ψ_W), and leaf osmotic potential were also determined in eight replicates per treatment. Total leaf C, N, H and S were determined using an elemental CHNS analyser (EA 1110 Automatic Elemental Analyser; Fisons EA-1108).

Plant extracts were obtained for the measurement of superoxide dismutase (SOD) and peroxidase (POD) activities. Five replicates per treatment were used for leaf as well as root analyses. Superoxide dismutase (SOD) activity was measured by the nitroblue tetrazolium reaction (*7*). Peroxidase (POD) activity was measured using guaiacol as hydrogen donor (*8*). The level of lipid peroxidation was measured by the thiobarbituric acid reaction (*9*) with slight modifications (*10*). Leaves and roots from five replicates per treatment were employed in these measurements. Sulfhydryl groups were determined in roots from five living plants per treatment (*11*).

ATPase activity was measured (*12*, *13*) in leaf and root microsomal fractions from five plants per treatment. Microsomal fractions were obtained (*14*). Protein content of this fraction was assayed by modified Bradford method (*15*, *16*). Total, plasma membrane, vacuolar, mitochondrial, and organular ATPase activities were measured using inhibitors. Six repetitions were tested for

Downloaded by PENNSYLVANIA STATE UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch004

each case. After hydrolysis, released inorganic phosphate was measured (*17*). Leaves from five replicates per treatment were used for free proline content determination (*18*). Soluble polyamines were determined (*19*, *20*).

Results and discussion

Plants treated with 1 mM BOA (BOA from now on), 60 mM NaCl (Salt from now on) and a combination of both treatments: 1mM BOA + 60 mM NaCl (BOA+Salt from now on) were less developed than control plants after 15 days exposition. These effects were reflected in the analysis of biometric data, where all treatments showed significant differences on plant height when compared to its corresponding control (Figure 1).

	Control	BOA	BOA+Salt	Salt
Plant height	a	b ***	b ***	b **
Root length	a	b *	b *	a n.s.

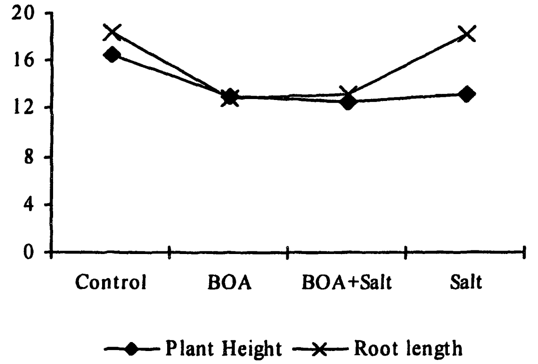


Figure 1. Height and root length (cm) of lettuce plants treated for 15 days with Hoagland's solution (control); 1 mM BOA (BOA), 60 mM NaCl (Salt), and 1 mM BOA + 60 mM NaCl (BOA+Salt).

*In the table, asterisks show significant differences when compared to control, whilst letters show significant differences between treatments (***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$; n.s.≥ 0.05).*

The height reduction was correlated also with the data of leaf biomass and necromass (Figure 2), where highly significant differences revealed inhibition of growth, as well increasing of senescence. The most affected plants were the lettuce plants exposed to the combined treatment (BOA+Salt), where the leaf biomass was 50% less than the control biomass.

Downloaded by PENNSYLVANIA STATE UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch004

Downloaded by PENNSYLVANIA STATE UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch004

Leaf	Control	BOA	BOA+Salt	Salt
Biomass	a	b c ***	c ***	b ***
Necromass	a	b ***	b ***	b **
Proteins	a	b *	a n.s.	a n.s.

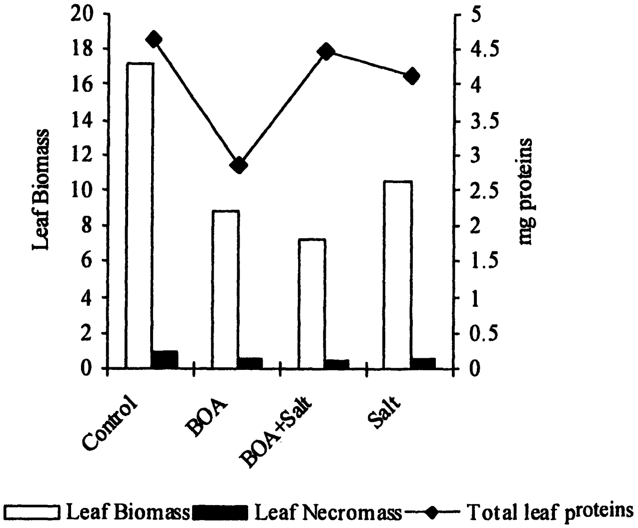
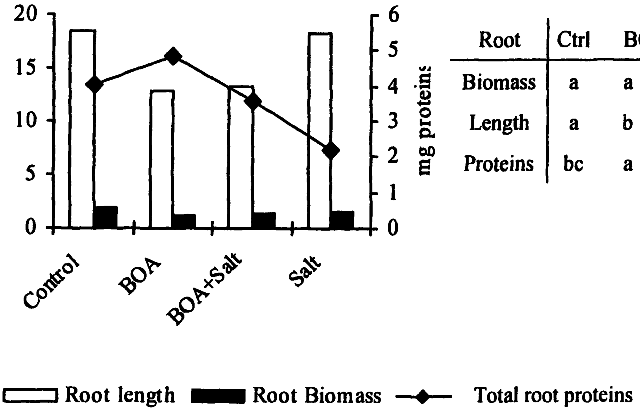


Figure 2. Leaf biomass (g), Leaf necromass (g) and Total leaf proteins (mg g^{-1} DW) in lettuce plants treated for 15 days with Hoagland's solution (control); 1 mM BOA (BOA), 60 mM NaCl (Salt), and 1 mM BOA + 60 mM NaCl (BOA+Salt).

*In the table, asterisks show significant differences when compared to control, whilst letters show significant differences between treatments (***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$; n.s.≥ 0.05).*

By contrary, the effects on root morphology seem to be specific for BOA exposition. In this way, root length (Figure 3) was only inhibited in presence of BOA, with very similar values for BOA and BOA+Salt treatments. As detected for BOA treatment, also the data of BOA+Salt showed plants with shorter (significant inhibition of root length) and thicker roots (same biomass with less root area) than control plants. It could be the consequence of BOA effects on cell wall extensibility, which can difficult elongation, growth, and development of lettuce roots.

It is remarkable that for leaf as well as for root protein contents the results show a clear specificity for the single BOA treatment, without significant differences in the combined treatment BOA+Salt. The reduction of total soluble leaf proteins could be the consequence of the strong increase on lipid peroxidation in the plants treated only with BOA, which is only significant for the single BOA treatment and that has been related with high levels of denatured proteins (data not shown). In the same way, in plants senescencing have shown a reduction in their protein content (*21*) by increasing proteolysis as well by reducing protein synthesis (*22*). Reduced protein synthesis has been previously found for other allelochemical compounds (*23*).



Root	Ctrl	BOA	BOA+Salt	Salt
Biomass	a	a n.s.	a n.s.	a n.s.
Length	a	b *	b *	a n.s.
Proteins	bc	a n.s.	c n.s.	b *

Figure 3. Root biomass (g), Root length (cm) and Total root proteins (mg g^{-1} DW) in lettuce plants treated for 15 days with Hoagland's solution (control); 1 mM BOA (BOA), 60 mM NaCl (Salt), and 1 mM BOA + 60 mM NaCl (BOA+Salt).

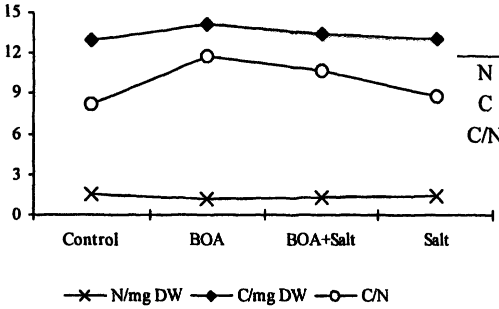
*In the table, asterisks show significant differences when compared to control, whilst letters show significant differences between treatments (***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$; n.s.≥ 0.05).*

There is an increase in C:N ratio when BOA is present in the medium (Figure 4). This increase is the result, in both treatments (BOA and BOA+Salt), of increased total carbon content and decreased total nitrogen content, commonly expected in stress situations. Stressed plants usually suffer decreases in nitrogen concentrations and increases in carbon-based secondary metabolites (*24*). The higher total C content suggests an increased-lipid production and a thicker cuticle, whereas a diminution in N content can imply a decrease in amino acids, proteins, and chlorophyll in these plants. However, salt treatment did not show significant differences in any of these parameters (data not shown).

Several ions have been measured (data not shown), and there is an effect of Na^+ on plant metabolism, with more drastic consequences on plant metabolism of BOA+Salt-treated plants, which suggests again a synergic effect of both stresses. This occurs even when NaCl concentration is higher in Salt-treated plants. The physiological effects of salt on plant metabolism can be observed on

Downloaded by PENNSYLVANIA STATE UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch004

Downloaded by PENNSYLVANIA STATE UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch004

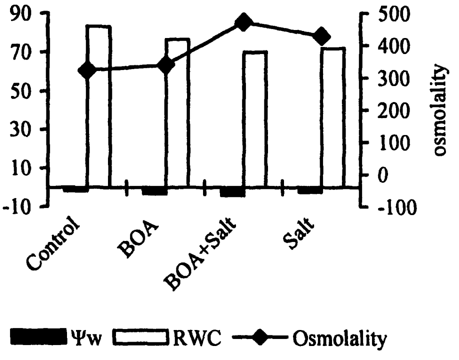


	Ctrl	BOA	BOA + Salt	Salt
N	a	b ***	b ***	a n.s.
C	a	c ***	b *	a n.s.
C/N	a	c ***	b ***	a n.s.

Figure 4. Concentration of total Nitrogen (N), total Carbon (C) and C/N ratio in lettuce leaves treated for 15 days with Hoagland's solution (control); 1 mM BOA (BOA), 60 mM NaCl (Salt) and 1 mM BOA + 60 mM NaCl (BOA+Salt).

In the table, asterisks show significant differences when compared to control, whilst letters show significant differences between treatments ($P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$; n.s.≥ 0.05).***

different essential processes, and the reaction of plant to this stress is usually directed to restoring ion homeostasis by lowering osmotic potential (Figure 5). Accumulating Na^+ in the vacuoles as well as increasing the production and accumulation of compatible osmolytes in the cells is a common strategy found in plants under salt stress. As shown in Figure 5, the osmolality values for plants under salt exposure are much lower than for control plants, with higher values in



	Ctrl	BOA	BOA+Salt	Salt
RWC	a	b **	c ***	bc ***
Ψw	a	b **	b **	a n.s.

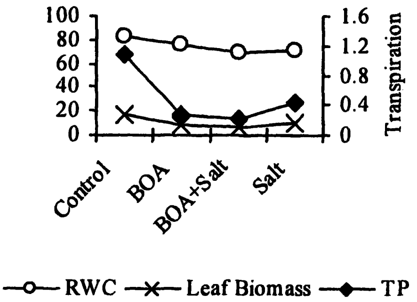
Figure 5. Relative water content (RWC, %), Water potential (Ψw, MPa) and Osmolality (Osm, mmol Kg^{-1}) in lettuce plants treated for 15 days with Hoagland's solution (control); 1 mM BOA (BOA), 60 mM NaCl (Salt), and 1 mM BOA + 60 mM NaCl (BOA+Salt).

In the table, asterisks show significant differences when compared to control, whilst letters show significant differences between treatments ($P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$; n.s.≥ 0.05).***

Downloaded by PENNSYLVANIA STATE UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch004

the combined BOA+Salt treatment. This supports an active strategy of the plant in restoring cell homeostasis. These results are directly correlated with results in RWC but not in Ψw. Although RWC and Ψs are lower in plants treated with salt, Ψw showed no significant differences for Salt treatment but severely lower values in plants exposed to BOA.

Compared to control, the decrease in RWC was correlated with a drastic decline in transpiration rate for the three treatments (Figure 6). This was most pronounced in the BOA treatment, where a strategy for limiting water loss by limiting transpiration seems clear. The stronger inhibition of transpiration rate when exposed to BOA could also reduce the salt uptake in the BOA+Salt treatment when compared to the Salt treatment (previously discussed). Since sodium uptake has been found to be proportional to the plant transpiration (*25*), an inhibition in transpiration rate as consequence of the effects generated by BOA on plant metabolism, results in less plasticity and turgor loss, implicating an adaptive strategy to salinity as a secondary but positive effect of the exposure of the plant to BOA.



	Cntrl	BOA	BOA+Salt	Salt
RWC	a	b **	c ***	bc ***
Biomass	a	bc ***	c ***	b ***
TP	a	b ***	b ***	b ***

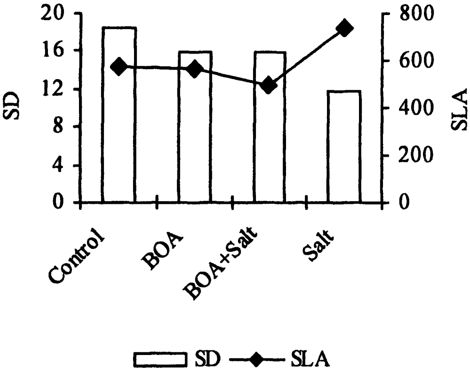
Figure 6. Relative water content (RWC, %), Leaf biomass (g) and Transpiration rate (TP, mol m^{-2} s^{-1}) in lettuce plants treated for 15 days with Hoagland's solution (control); 1 mM BOA (BOA), 60 mM NaCl (Salt), and 1 mM BOA + 60 mM NaCl (BOA+Salt).

*In the table, asterisks show significant differences when compared to control, whilst letters show significant differences between treatments (***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$; n.s.≥ 0.05).*

Growth under stress conditions has been previously related to increases in specific leaf area (SLA, Figure 7) (*26*). Salt treated plants showed an increase in SLA and a highly significant decrease in stomatal density (SD). This could be a new indication about a major ability of Salt-treated plants to tolerate salt stress by a strategy for minimising salt effects on plant metabolism. This tolerant behaviour is correlated with the high capacity for osmoregulation. Proline concentration (Figure 8) seems one of the main osmoregulators. Concentration

Downloaded by PENNSYLVANIA STATE UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch004

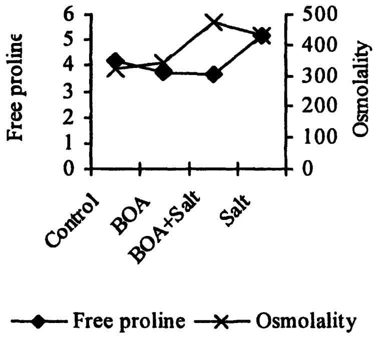
of this osmolyte has been considered to be a determinant criterion to study plant tolerance to salt stress (*27*). Proline minimises the impact of abiotic stresses on plants (*18*). Proline accumulation can be the consequence of an increase on its production as well as a decrease on its degradation (*28*). Our data suggest that proline accumulation is not due to degradation, because no correlation was found between free proline content and total soluble proteins in these plants (Figure 9).



	Cntrl	BOA	BOA+Salt	Salt
SD	a	b *	b *	c ***
SLA	a	a n.s.	a n.s.	b **

Figure 7. Stomatal density (SD, number per field) and Specific leaf area (SLA, m^2 g^{-1} DW) in lettuce plants treated for 15 days with Hoagland's solution (control); 1 mM BOA (BOA), 60 mM NaCl (Salt), and 1 mM BOA + 60 mM NaCl (BOA+Salt).

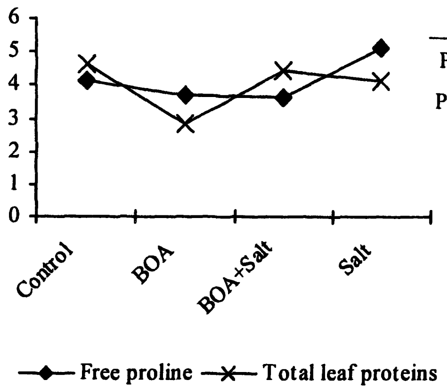
In the table, asterisks show significant differences when compared to control, whilst letters show significant differences between treatments ($P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$; n.s.≥ 0.05).***



	Control	BOA	BOA+Salt	Salt
Proline	a	a n.s.	a n.s.	b **
Osm.	a	a n.s.	c ***	b ***

Figure 8. Free proline (µmol g^{-1} DW) and Osmolality (Osm, mmol Kg^{-1}) in lettuce plants treated for 15 days with Hoagland's solution (control); 1 mM BOA (BOA), 60 mM NaCl (Salt), and 1 mM BOA + 60 mM NaCl (BOA+Salt).

In the table, asterisks show significant differences when compared to control, whilst letters show significant differences between treatments ($P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$; n.s.≥ 0.05).***



	Cntrl	BOA	BOA+Salt	Salt
Proline	a	a n.s.	a n.s.	b **
Proteins	a	b *	a n.s.	a n.s.

Figure 9. Free proline (µmol g^{-1} DW) and Total leaf proteins (mg g^{-1} DW) in lettuce plants treated for 15 days with Hoagland's solution (control); 1 mM BOA (BOA), 60 mM NaCl (Salt), and 1 mM BOA + 60 mM NaCl (BOA+Salt).

In the table, asterisks show significant differences when compared to control, whilst letters show significant differences between treatments (***P≤0.001; **P≤0.01; *P≤0.05; n.s.≥0.05).

In the BOA+Salt treatment, where osmolality values are especially high, and where strategies must take place also for restoring ion homeostasis, there was not a significant increase in free proline content. As shown in Figure 10, the osmolality value in this treatment is related to a severe increase in soluble putrescine content. While free proline results suggest that a salt-tolerant strategy is taking place in Salt-treated plants, in previous works the increments in putrescine under salt stress were typically found in salt-sensitive cultivars (*29*), where putrescine catabolism contributed to compatible osmolyte accumulation. Nevertheless, even when previous research has suggested that increases in putrescine content can reduce inhibited plant growth, our data suggest that there is no correlation between both parameters, because plant height and biomass of BOA+Salt treated plants were both severely inhibited (see Figsures 1 and 2).

As expected, the previously discussed decreases in transpiration rate and the reductions in RWC in plants treated with BOA, Salt and BOA+Salt were correlated with highly significant decreases of net photosynthetic rates in the three treatments. Once more, the most affected treatment was the combined BOA+Salt, with photosynthetic rates around 50% of the control. The interactions between plant water relations and photosynthetic gas exchange measurements seem clear (*30*).

All treatments have higher Water Use Efficiency (WUE) values when compared to the control, as result of drastic inhibition of the photosynthetic process and the strong reduction of transpiration. It is remarkable here, that even with very similar values of net Carbon photosynthesis (P_n), the strategy of behaviour in BOA plants and in Salt plants is quite different since WUE in

Downloaded by PENNSYLVANIA STATE UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch004

BOA-treated lettuce is much higher than in plants treated with salt. Gas-exchange measurements and chlorophyll fluorescence values were recorded for all treatments. BOA+Salt caused the most drastic effects on the photosynthetic process with the strongest inhibition in P_n, photochemical yield of PSII (Fv/Fm ratio), and quantum yield of electron flow through PSII *in vivo* (Φ_{PSII}). This last parameter (Φ_{PSII}) that was highly significant in BOA+Salt plants represents the overall efficiency of PSII reaction centres in the light and it is typically affected by photoinhibition (*31*). Φ_{PSII} measures the proportion of absorbed energy that is used in photochemistry, therefore, its inhibition suggests an alteration in electron transport. At the same time, the Fv/Fm values reveal a physical damage in the photosynthetic apparatus, since the inhibition of Fv/Fm suggests weak damages at PSII reaction centres in BOA+Salt treated plants. The over reduction of the photosynthetic electron transport chain (here detected for BOA and BOA+Salt treated plants) has been previously observed in several environmental stresses as a consequence of a limited CO_2 fixation under stress and the concomitant reduction in $NADP^+$ regeneration by the Calvin cycle (*32*). In this way, the lower transpiration rate could indicate that stomatal closure as well as the significant reduction of stomatal density (see Figure 7) are ocurring in the three treatments.

	Cntrl	BOA	BOA+Salt	Salt
Proline	a	a n.s.	a n.s.	b **
PUT	a	a n.s.	b ***	a n.s.

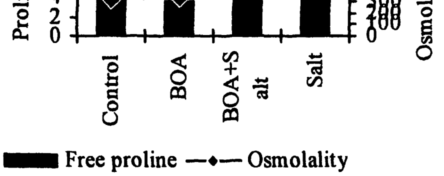


Figure 10. Free proline (μmol g^{-1} DW), soluble putrescine (PUT, nmol g^{-1} DW) and osmolality (Osm, mmol Kg^{-1}) in lettuce plants treated for 15 days with Hoagland's solution (control); 1 mM BOA (BOA), 60 mM NaCl (Salt), and 1 mM BOA + 60 mM NaCl (BOA+Salt).

In the table, asterisks show significant differences when compared to control, whilst letters show significant differences between treatments ($P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$; n.s.≥ 0.05).***

Downloaded by PENNSYLVANIA STATE UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch004

This stomatal behaviour could explain the inhibition of photosynthesis as the result of a reduction in CO_2 assimilation, which is usually found in plants exposed to water deficit, excess of salt, low and high temperature, etc. When there is an increase of non-photochemical quenching as consequence of an excess of energy in the chloroplasts as we found here, it can lead to the stimulation of alternative processes for energy dissipation like heat radiation, xanthophyll cycle-dependent energy dissipation, and the production of reactive oxygen species (ROS) such as superoxide and H_2O_2.

As a consequence of the deleterious nature of some of these ROS, plants have developed a variety of antioxidant systems (enzymatic and non-enzymatic) for their detoxification. The antioxidant reactions avoid toxicity by maintaining the redox balance of the cell; but when antioxidant systems do not work correctly, ROS accumulation takes place, and concomitantly cell injury and plant development inhibition can appears too. Although research showing increases in the activities of antioxidant enzymes (SOD, POD) under salt stress are abundant in the literature (*33*, *34*), we have not found a positive correlation between salt or BOA stress and these enzymatic activities at the leaf level. On the contrary, our data show a drastic inhibition on leaf SOD activity for the three treatments, and a BOA-induced leaf POD activity inhibition in BOA and BOA+Salt-treated plants. This implies an increase on ROS content resulting in cytotoxic protein damage, DNA damage, and lipid peroxidation. The lower SOD activity could be the consequence of the action of free radicals on enzymatic denaturation, as well as a consequence of senescence processes taking place in the plant after severe stress exposure.

The significant decline of leaf SOD and POD activities was correlated with a significant increase of malondialdehyde content (MDA) in the plants of the single BOA-treatment. MDA is a typical marker of lipid peroxidation, which reflects an increase in free radical content that would damage the lipid composition of the membrane, altering their properties and inducing severe damage to the cell. On the contrary, with salt-treated plants (from single as well as combined treatment) there was not a significant increase on MDA content. These data are in accordance with those found (*35*), which suggest that Salt-treated plants don't suffer oxidative damage (perhaps because 60 mM NaCl is a too low concentration for producing secondary stress, and plants are able to compensate it) or the plants are able to enhance the detoxification of ROS via non-enzymatic antioxidants such as ascorbate, carotenoids or α-tocopherol. These antioxidant constituents have been found to be induced by ABA accumulation under conditions of water stress, as usually happens under salinity (*36*). When data of root SOD and POD activities are analysed (data not shown) results are different. In this case, POD activity is not affected by BOA or Salt stress when compared to control. The only affected enzyme in lettuce roots was SOD activity, which is reduced 50% in BOA-treated plants than in the

Downloaded by PENNSYLVANIA STATE UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch004

corresponding control. The only treatment with significant lipid peroxidation was BOA treatment. The increase of MDA content is positively correlated with increased free sulfhydryl groups in this treatment (data not shown). In this way, ROS-induced injury, which causes peroxidation of lipid membranes, can also cause oxidation of protein thiol groups and membrane depolarization. These protein modifications can be either by direct oxidation or by modifications mediated by products of lipid peroxidation. The increase on soluble sulfhydryl groups can be the result of ROS-induced damage to protein structure and function.

BOA+Salt and Salt treatments caused neither lipid peroxidation nor the related increase in sulfhydryl groups. This is a sign that these plants are not suffering oxidative damage and that they are developing antioxidants mechanisms to compensate for the production of ROS as consequence of stress.

Finally, polyamines contents were analised (data not shown). Spermine (SPM), spermidine (SPD), and putrescine (PUT) contents seem to be altered by the presence of BOA in the medium. The increased PUT content in the combined treatment BOA+Salt has been previously discussed to be related with tolerance mechanisms to salt stress by allowing this diamine to lower the osmotic potential and so better support the turgor lost in these plants (*37*). The decrease in SPM and SPD levels could contribute to the oxidative burst due to the important protective role that these polyamines play in the plasma membrane of the cells. Concomitantly, this oxidative burst could initiate signal transduction and lead to the activation of defence mechanisms at the initiation of stress (*38*), in this case to BOA action. The higher PUT content as well as the decline in SPM and SPD contents in both treatments (BOA and BOA+SALT) result in a significant decrease of the index Pas/Das (ratio polyamines to diamines), usually employed as stress marker.

Downloaded by PENNSYLVANIA STATE UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch004

Conclusions

BOA+Salt-treated plants had the lowest photosynthetic capacity and, concomitantly, a drastic decrease on plant growth and root length when compared to control plants and also with plants from the single stress treatments. Combined stresses (BOA+Salt) did not imply a combined response in these plants, since the response in BOA+Salt-treated plants was totally different to the response found in plants treated only with BOA or with Salt. The mechanisms of both stresses seem related at least in part with a disruption of the plant water balance, with altered RWC in all the treatments. There was oxidative damage in plants after 15 days BOA exposure, with high levels of lipid peroxidation and sulfhydryl groups, and effects on essential enzymatic activities such as SOD, POD and even ATPases. Salt-treated plants showed osmoregulation dominance.

In plants exposed for 15 days to the combined treatment 1 mM BOA and 60 mM NaCl no clear oxidative damage or osmoregulation was observed. However, the data reveal that there is a group of altered processes in BOA+Salt treated plants without relation with the mode of action proposed for BOA or for salt-stressed metabolism. These plants show "classical" salt-sensitive signals. This suggests that the presence of the allelochemical reduces the capacity of the plant to use classical tolerance mechanisms against salt stress.

References

1. Sánchez-Moreiras, A. M. Whole Plant Stress Response After BOA Exposition, Dep. Bioloxía Vexetal e Ciencia do Solo, Universidade de Vigo, Spain, 2004.
2. Chiapusio, G.; Sánchez-Moreiras, A. M.; Reigosa, M. J.; González, L.; Pellissier, F. *J. Chem. Ecol.* **1997**, *23*, 2445-2453.
3. Reigosa, M. J.; González, L.; Sánchez-Moreiras, A. M.; Durán, B.; Puime, O.; Fernández, D. A.; Bolaño, J. C. *Allelopathy Journal* **2001**, *8*, 211-220.
4. Reigosa, M. J.; Pedrol, N.; Sánchez-Moreiras, A. M.; González, L. In *Allelopathy: From Molecules to Ecosystems*; Reigosa, M. J.; Pedrol, N., Eds.; Science Publishers, Inc.: Enfield, NH, 2002; pp 231-256.
5. Rausch, T.; Kirsch, M.; Low, R.; Lehr, A.; Viereck, R.; Zhigang, A. *J. Plant Physiol.* **1996**, *148*, 425-433.
6. Hoagland, D. R,.; Arnon, D. J.; *California Agricultural Experiment Station Circular* 347, 1950.
7. Beauchamp, C.; Fridovich, I. *Anal. Biochem.* **1971**, *44*, 276.
8. Silva, E.; Nogueira, J. N. *O Solo* **1984**, *76*, 43-51.
9. Heath, R.L.; Packer, L. *Arch. Biochem. Biophys.* **1968**, *25*, 189-198.
10. Dhindsa, R. S.; Plumb-Dhindsa, P.; Thorpe, T. A. *J. Exp. Bot.* **1981**, *32*, 93-101.
11. Prudencio-Ferreira, S. H.; Areas, J. A. G. *J. Food Sci.* **1993**, *58*, 378-381.
12. Calera, M. R.; Anaya, A. L.; Gavilanes-Ruiz, M. *J. Chem. Ecol.* **1995**, *21*, 289-297.
13. Cruz-Ortega, R.; Anaya, A. L.; Gavilanes-Ruiz, M.; Sánchez Nieto, S.; Jiménez Estrada, M. *J. Chem. Ecol.* **1990**, *16*, 2253-2261.
14. Flores, H. E. In *Biochemistry and Physiology of Polyamines in Plants*; Slocum, R. D.; Flores, H. E., Eds.; CRC Press: Boca Raton, FL, 1991; pp 213-226.
15. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248-254.
16. Stoscheck, C. M. *Methods Enzymol.* **1990**, *182*, 50-69.
17. Chifflet, S.; Torriglia, A.; Chiesa, R.; Tolosa, S. *Anal. Biochem.* **1998**, *168*, 1-4.

Downloaded by PENNSYLVANIA STATE UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch004

18. Ramos, P.; Pedrol, N. In *Handbook of Plant Ecophysiology Techniques*; Reigosa, M. J., Ed.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2001; pp 365-382.
19. Pedrol, N.; Tiburcio, A. F. In *Handbook of Plant Ecophysiology Techniques*; Reigosa, M. J., Ed.; Kluwer Academic Publishers, Dordrecht, The Netherlands, 2001; pp 335-364.
20. Reggiani, R.; Hochkoeppler, A.; Bertani, A. *Plant Physiol.* **1989**, *91*, 1197-1201.
21. Watkinson, A. *Trends Ecol. Evol.* **1992**, 7, 417-420.
22. Wen, J. Q.; Tan, B. C.; Liang, H. G. *J. Plant Physiol.* **1996**, *148*, 707-710.
23. Cameron, H. J.; Julián, G. R. *J. Chem. Ecol.* **1980**, *6*, 989-995.
24. Chapin, F. S. III; Shaver, G. R.; Kedrowski, R. A. *J. Ecol.* **1986**, *4*, 167-195.
25. Ash, F.; Dingkuhn, M.; Dörffling, K. *J. Exp. Bot.* **1997**, *48*, 39.
26. Leidi, E. O.; Saiz, J. F. *Plant Soil* **1997**, *190*, 67-75.
27. Yoshiba, Y.; Kiyosue, T.; Nakashima, K.; Yamaguchi-Shinozaki, K.; Shinozaki, K. *Plant Cell Physiol.* **1997**, *38*, 1095-1102.
28. Mattioni, C.; Lacerenza, N. G.; Troccoli, A.; De Leonardis, A. M.; Di Fonzo, N. *Physiol. Plant.* **1997**, *101*, 787-792.
29. Krishnamurthy, R.; Bhagwat, K. A. *Plant Physiol.* **1989**, *91*, 500-504.
30. Nagy, L.; Balint, E.; Barber, J.; Ringler, A.; Cook, K. M.; Maroti, P. *J. Plant Physiol.* **1995**, *145*, 410–415.
31. Epron, D.; Dreyer, E.; Breda, N. *Plant Cell Environ.* **1992**, *15*, 809–820.
32. Vranová, E.; Inzé, D.; van Breusegem, F. *J. Exp. Bot.* **2002**, *53*, 1227-1236.
33. Hernández, J. A.; Jimenez, A.; Mullineaux, P.; Sevilla, F. *Plant Cell Environ.* **2000**, *23*, 853-862.
34. Wang, B.; Lüttge, U.; Ratajczak, R, *J. Exp. Bot.* **2001**, *52*, 2355-2365.
35. Lu, C. M.; Qiu, N. W.; Lu, Q. T.; Wang, B. S.; Kuang, T. Y. *Plant Sci.* **2002**, *163*, 1063-1068.
36. Jiang, M.; Zhang, J. *Free Rad. Res.* **2002**, *36*, 1001-1015.
37. Pedrol, N.; Ramos, P.; Reigosa, M. J. *J. Plant Physiol.* **2000**, *157*, 383-393.
38. Smith, T. A. *Annu. Rev. Plant Physiol.* **1986**, *36*, 117-143.

Downloaded by PENNSYLVANIA STATE UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch004

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch005

Chapter 5

Chemical and Biological Characterization of Toxins Produced by Weed Pathogenic Fungi as Potential Natural Herbicides

Antonio Evidente

**Dipartimento di Scienze del Suolo, della Pianta e dell'Ambiente,
Università di Napoli Federico II, Via Università 100, 80055 Portici, Italy**

Several phytopathogenic fungi have been proposed for biocontrol of weeds, but few have been used. Recent studies were conducted to develop integrated biocontrol strategies using a combination of low-dose mycoherbicides and the phytotoxins they produce. This chapter describes the chemical and the biological characterization of phytotoxins produced by some *Ascochyta*, *Pyrenophora* and *Phoma* species proposed for the biocontrol of weeds (fat hen, annual grasses and sowthistle) infesting important agrarian crops, such as maize, sugar beet and cereals. Furthermore, the importance of phytotoxins in developing integrated weed control strategies is discussed.

Introduction

Weeds are one of the most serious problems for agriculture and the environment. Infesting plants obstruct the normal flow of surface waters, alter the natural habitat, and cause heavy losses to crop production and the pasture industry. The control of weeds has been achieved with synthetic herbicides. They are usually used in large amounts in agriculture, causing problems to

In Natural Products for Pest Management; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

human and animal health, and environmental pollution. Therefore, many efforts have been made to biologically control weeds, using their natural antagonists such as insect s and/or microorganisms.

Biological agents offer the advantage of being compatible with the environment, often with high specificity, and also represent a long term solution in the control of weeds with resistance to chemical herbicides.

Recently, research was begun to isolate phytotoxins produced by some pathogenic fungi for weeds and on their use as natural herbicides. The goal of such studies is to use natural substances, their derivatives or synthetic analogues with increased efficacy and specificity to avoid microorganism release, and the possibility that they became pathogens of other organisms (*1-5*). Furthermore, the phytotoxins could be used indirectly as biomarkers for the improvement of fungi mycoherbicide properties.

Phytotoxins are defined as microbial metabolites that are harmful to plants at low concentrations. These toxins often have low molecular weights and belong to several classes of natural products. The virulence of plant pathogens may depend on its capability to synthesize one or more toxins. Only few phytotoxins are known as host-specific toxins, and they are often phytotoxic to a broad range of plant species. In some cases, studies on their mode of action and their role as a "vivo-toxin" have also been carried out (*4*, *6-9*).

Ascochyta caulina phytotoxins

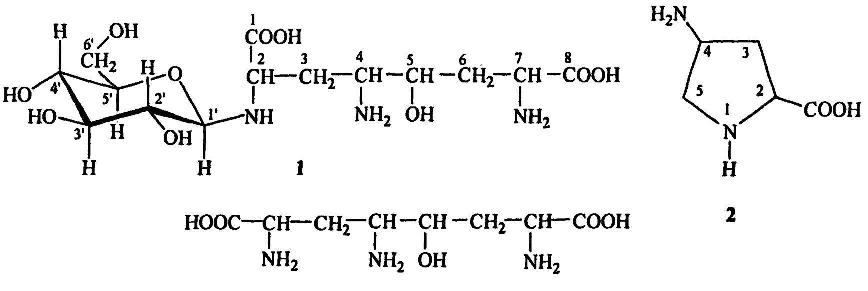
Ascochyta caulina (P. Karst.) v.d. Aa and v. Kest. is a promising mycoherbicide for the control of *Chenopodium album* (*10*), also known as common lambsquarters or fat hen, a world-wide diffused weed of arable crops such as sugar beet and maize throughout the world (*11*).

Isolation and Chemical Characterization of the Phytotoxins

The phytotoxins produced *in vitro* by a standard strain of *Ascochyta caulina* (AC-1, kindly supplied by Dr. Piet Scheepens, Plant Research International, Wageningeen, The Netherlands) were purified by combination of cationic-exchange, gel filtration and reversed phase TLC. Three toxins were isolated and characterized using spectroscopic and chemical methods. The main toxin, named ascaulitoxin (235 mg/Li, **1**, Figure 1) appeared to be a N^2-β-D-glucopyranoside of the 2,4,7-triamino-5-hydroxyoctandioic acid (*12*). The other two toxins, which are non-proteinogenic amino acids like **1**, were characterized as *trans*-4-amino-D-proline and ascaulitoxin aglycone (150 and 150 mg/L, **2** and **3**, respectively, Figure 1) (*13-14*).

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch005

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch005



1

2

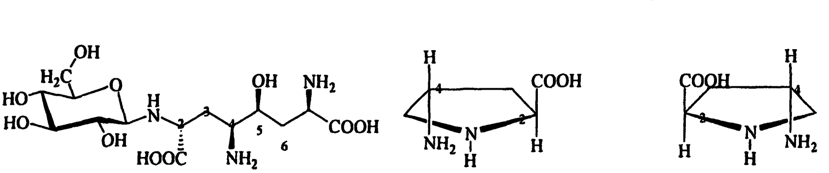
3

Figure 1. Ascochyta caulina *phytotoxins.*

Any practical application of ascaulitoxin as a natural herbicide appears to be seriously limited by the very low amounts of this metabolite present in the fungal culture filtrates. Therefore, efforts must be first directed towards devising a convenient and simple method for total synthesis. The first attemps to realize this goal in now in progress.

The preliminary determination of the relative configuration of the four chiral centers (C-2, C-4, C-5 and C-7) of ascaulitoxin (**1**) appears to be relevant to establish its absolute stereochemistry and to realize its stereoselective synthesis.

The determination of the relative stereochemistry of ascaulitoxin molecule (**1**) was performed by NMR configurational analysis, based on the evaluation of the homo ($^3J_{H\text{-}H}$) and hetero ($^2J_{C\text{-}H}$ and $^3J_{C\text{-}H}$) nuclear coupling constants, in combination with ROESY (Rotating Overhauser Effect Spectroscopy) responses. This represents a success in the application of this method to the nitrogen-substituted chains. The conformation, and the relative stereochemistry of the four chiral centres of ascaulitoxin was determined as in **4** (Figure 2) (*15*).



4

5 [*trans*-L-Enantiomer (*2S,4R*)]

6 [*trans*-D-Enantiomer *(2R,4S)*]

Figure 2. The relative stereochemistry of ascaulitoxin (4) and 4-aminoproline enantiomers (5 and 6).

The absolute stereochemistry of **2** was achieved using chemical and spectroscopic methods. In fact, the toxin was converted to its N^1,N^4-ditosyl methyl ester derivative, which showed different spectroscopic and chromatographic behaviour when compared to the analogue derivative of the *cis*-

4-amino-L-proline. The latter was synthesized from *trans*-4-hydroxy-L-proline, the natural amino acid, according to reaction sequence already reported (*16*). Therefore, the toxin is the D-enantiomer (**6**, Figure 2) of the *trans*-4-aminoproline, because an opposite optical rotation, with respect to that reported for the *trans*-4-amino-L-proline (**5**, Figure 2), was recorded (*14*).

Biological Characterization of Phytotoxins

When assayed at 30 μg/droplet on punctured leaves of host and non-host weed and cultivated plants, ascaulitoxin showed a different degree of phytotoxicity on tested species (Table 1). Ascaulitoxin caused the appearance of necrotic spots surrounded by chlorosis on the leaves of fat hen. Clear necrosis also appeared both on weeds (common sowthistle, annual fleabane noogoora burr, tree of heaven) and on cultivated plants (pea and cucumber). Clear, but in reduced size, were necrosis on tomato and redroot pigweed (*12*).

More intresting results were observed in testing the *trans*-4-amino-D-proline due to its selective phytotoxicity against dicot plants. Assayed at 1 mg/μl on punctured leaves on the same plants (Table 1), the toxin (**2**) had a drastic effect on the host plant, causing the rapid appearance of a large necrosis area surrounding the puncture point. The toxin was active at a concentration 5 times lower, but caused less necrosis. On other dicot leaves, the phytotoxicity varied from large necrotic areas (poppy, annual mercury, cucumber, wild cucumber), through medium ones (tree of heaven, tomato, common sowthistle) to small necrotic spots (black nightshade). An interesting aspect was the lack of toxicity of **2** when assayed on several monocots, both cultivated (wheat, oat, barley) as well as wild (canarygrass, slender foxtail, wild oat). When tested at up to 10^{-5} M on cut young fat hen seedlings, the toxin caused wide necrosis and dryness of cotyledons, while no effects could be seen on stems (*13*).

Toxins **1** and **2** lack antifungal and antibiotic activities when assayed (up to 50-100) μg/disk on *Geotrichum candidum*, *Pseudomonas syringae* subsp. *syringae* and *E. coli*, and they have no zootoxicity when tested at up to 40 μg/ml sea solution on brine shrimp larvae (*Artemia salina* L.) (*12-13*).

Greenhouse and Field Experiments

An alternative method based on ion exchange chromatography was developed to overcome difficulties and high cost to obtain each *A. caulina* toxin in a pure form. In fact, after mild acidification (formic acid) the culture filtrates were loaded on a cation-exchange chromatographic (Dowex 50, H^+ form) column and water eluted to collect saccharose, which was used as carbon source in the culture medium, and other non basic compounds. Successive elution with

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch005

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch005

Table 1. The phytotoxicity of ascaultioxin and *trans*-4-amino-D-proline (1 and 2) by leaf puncture assay on host and non-host plants[a]

Latin name	Common name	Effect on Leaves[b] 1	Effect on Leaves[b] 2
Ailanthus glandulosa	Tree of Haeven	++	++
Alopecurus myosuroides	Slender foxtail	NT	-
Amaranthus retroflexus	Redroot pigweed	+	NT
Avena fatua	Wild oat	NT	-
Avena sativa	Oat	NT	-
Chenopodium album	Fat hen	+++	++++
Cucumis sativus	Cucumber	++	+++
Ecballium elaterium	Wild cucumber	NT	+++
Erigeron annuus	Annual fleabane	++	NT
Hordeum vulgare	Barley	NT	-
Lycopersicon esculentum	Tomato	+	++
Mercurialis annua	Annual Mercury	NT	+++
Papaver rhoeas	Poppy	NT	+++
Phalaris canariensis	Canarygrass	NT	-
Pisum sativum	Pea	+	NT
Solarum nigrum	Black nightshade	NT	+
Sonchus oleraceus	Common sowthistle	++	++
Triticum durum	Durum wheat	NT	-
Xanthium occidentale	Noogoora burr	++	NT

[a] The toxins were assayed at concentration of 1.5 and 1 μg/μl for **1** and **2**, respectively. The speed of symptoms development varied between 2-5 days, depending on the species. [b] Toxicity index determined on the following scale: -, no symptoms; +, necrosis (0-1 mm); ++, necrosis (1-3): mm); +++, necrosis (3-5 mm); ++++, necrosis (6-7 mm). Results of at least three replicates. [c] NT=Not Tested.

ammonium hydroxide allowed collection of only a mixture of the toxic metabolites (350 mg/l) (*17*). The efficacy of the toxin mixture was compared with that of culture filtrates alone or in combination with the fungus. In glasshouse experiments it showed the same toxicity as culture filtrates. Greenhouse experiments on young fat-hen plants also showed that the use of the toxin mixture solution (1 mg/ml) in conjunction with spores of *A. caulina* (at 10^6/ml) improved the biocontrol efficacy of this fungus by more than 30%. Furthermore, the simultaneous application of toxins or fungal spores with low doses of herbicides, such as metribuzin and rimsulfuron, at 1/5 of labeled rate, gave better results than single-agent treatments (*19*).

Formulations containing different combinations of *A. caulina* conidia, its phytotoxins and low dose herbicides have been tested. A significant improvement in the efficacy of the fungus was achieved in glasshouse trials with an aqueous formulation containing PVA, a polyvinyl alcohol, Psyllium, a plant-derived polysaccharide, Sylgard 309, a surfactant, and nutrients and conidia (5 x 10^6/ml). Field trials have investigated the performance of *A. caulina* conidia applied at different development stages of *C. album*, either as a single treatment or combined with sub-lethal doses of herbicides or with the fungal phytotoxins. With the available formulations, favourable weather conditions are needed to obtain infection in the field (*18*).

Phytotoxin Analytical Methods and their Application as Biomarkers

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch005

A specific method for the qualitative and quantitative analyses of *A. caulina* toxins (**1**, **2** and **3**) by HPAC-PAD (High Performance Anionic Chromatography-Pulsed Amperometric Detector) on a Dionex chromatograph was developed (*14*). The toxins were well separated on an AminoPac PA1 analytical column. The mobile phase consisted of 23 mM sodium hydroxide and 7 mM sodium tetraborate solution. The developed method proved to be relatively simple, rapid, sensitive and reproducible.

This method, applied to the culture filtrates of different strains of *A. caulina* for the qualitative and quantitative analyses of toxins produced *in vitro* required the preliminary separation of saccharose by cation-exchange chromatography, because its peak overlapped that of ascaulitoxin. The three toxins were well separated and a recovery rate close to 100% was observed for all toxins (*14*). This method was applied to quantitatively estimate the toxin content in the culture filtrates of 10 *A. caulina* strains (AC-1, AC-2, AC-14, Ac-16, AC-18, AC-21, AC-22, AC-31, AC-35, Ac-46) from different origins. The data obtained showed that the strains synthesized all three toxins: two of them (AC-2 and AC-14) were higher producers than the standard strain. Only a strain (AC-46) was higher ascaulitoxin producer, with respect to the other toxins. It is also important to note to the wide range in toxin content between the tested culture filtrates of 74-298, 6-115, and 42-565 mg/l, for **1**, **2** and **3**, respectively, suggesting that there is no relation between the strain origin and their ability of to produce different toxins (*14*).

Pyrenophora and *Phoma* Phytotoxins

Among the possible causes of loss in cereal yields, annual grass weeds, are one of the most important; this is due to their similarity in morphology, physiology and ecology to the crop species. A feature common to annual grasses is their prodigious seed production, which is responsible for their reproduction and diffusion, even if their viability is low.

Methods to reduce the input of seed can improve long-term control of infesting grasses. One strategy is be the application of seed-borne pathogens as bioherbicides. Pathogens damaging the seed in the inflorescence or preventing flowering have also potential for biological control.

Pyrenophora semeniperda (Brittlebank & Adam) Shoemaker, a seed-borne pathogen that causes several symptoms in infected plants, has been proposed as a bioherbicide (*19*). The fungus infects seeds and leaves of over 35 genera of grasses including all winter cereals and six dicotyledonous genera (*20*). In brome grass (*Bromus* spp.) and wheat (*Triticum aestivum* L.) it has been reported to cause the death of seed primordia and the subsequent abortion of seed (*21*).

It is well known that other species of *Pyrenophora* produce toxins, some of which are potentially dangerous (*22-23*). Therefore, it seemed interesting to investigate the production of toxins by this species of *Pyrenophora*.

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch005

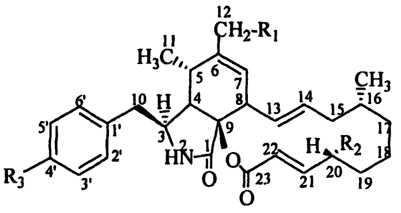
Isolation and Chemical Characterization of *P. semeniperda* Phytotoxins

Preliminary *in vitro* experiments showed that the fungus in liquid culture produces low-molecular lipophilic phytototoxins. The isolation, chemical and biological characterisation of these phytotoxins are in progress. When grown on wheat kernels *P. semeniperda* does not produce such phytotoxins but rather cytochalasins, a large group of fungal metabolites having different biological activities (*4*, *24-25*). This fungus synthesizes three new cytochalasins, named cytochalasins Z1, Z2 and Z3 (11, 11 and 1.6 mg/kg, **7**, **8** and **10**, respectively, Figure 3) together with four already known cytochalasins; cytochalasin B, produced in very large amounts (3.64 g/kg), cytochalasins F and T, and deoxaphomin (**13**, **15**, **9** and **16**, respectively, Figure 3) (*26*).

Cytochalasin Z1 represents the first example of a 24-oxa[14]cytochalasan bearing a *p*-hydroxybenzyl residue at C-3 of the perhydroisoindolyl-1-one moiety (*24-25*). Furthermore, cytochalasins Z1 and Z3, structurally related to cytochalasins T and B, respectively, are the first two 24-oxa[14]cytochalasans with a lactonic macrocyclic ring deoxygenated at C-20 (*26*). Cytochalasin Z2, closely related to the well known cytochalasin T, is a 24-oxa-[14]cytochalasan showing for the first time, among all the [11], [13] and [14]cytochalasans, a hydroxymethyl group on the C-6 (*26*).

Biological characterization of cytochalasins produced by *P. semeniperda*

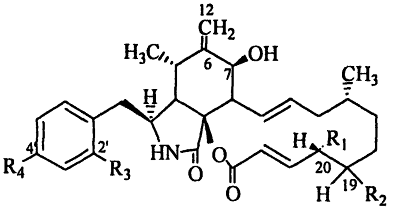
In seedling assays (Figure 4) on wheat and on tomato, the most active compounds were cytochalasin B (CB), its 21,22-dihydroderivative (diHCB), cytochalasins F and Z3 (CF and CZ3), and deoxaphomin (DEOXA). They were all able to reduce the root length by about 50%. In the puncture assay, only deoxaphomin, at the used concentration, showed the ability to produce small necrotic lesions. No effects were produced in the immersion assay by any of the tested cytochalasins (*26*). This, together with the observed phytotoxicity of liquid culture filtrates, could mean that other metabolites are responsible for phytotoxic effects caused by the pathogen.



7 $R_1=R_2=H$, $R_3=OH$

8 $R_1=R_2=OH$, $R_3=H$

9 $R_1=R_3=H$, $R_2=OH$

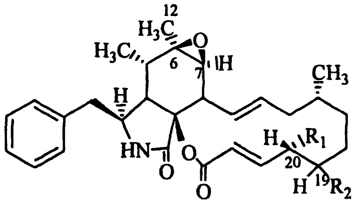


10 $R_1=R_3=R_4=H$, $R_2=OH$

11 $R_1=R_3=OH$, $R_2=R_4=H$,

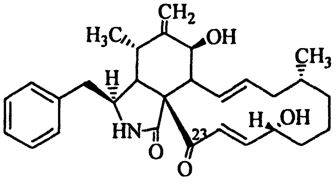
12 $R_1=R_4=OH$, $R_2=R_3=H$

13 $R_1=OH$, $R_2=R_3=R_4=H$



14 $R_1=H$, $R_2=OH$

15 $R_1=OH$, $R_2=H$



16

Figure 3. Pyrenophora and Phoma *phytotoxins.*

Further New Cytochalasins from *Phoma exigua* var. *heteromorpha*

Phoma exigua var. *heteromorpha* (Schulzer *et* Saacc.) Noordless *et* Boerema, the causal agent of a severe foliar disease of Oleander (*Nerium olender* L.) observed in 1985 in a nursery near Bari (Italy), proved to be a good producer of cytochalasins. From liquid and solid cultures of this fungus, several known (cytochalasins A, B and F and deoxaphomin) and new (ascochalasin, 7-O-acetylcytochalasin B, Cytochalasin T, U, V and W) cytochalasins were isolated, and their biological activities and SAR were investigated (*4*, *25*). Because the potential applications and the availability of large amount of solid cultures of *P. exigua* var. *heteromopha*, new cytochalasins were isolated from

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch005

this fungus. In fact, from its wheat kernel culture, three new cytochalasins were found and named Z4, Z5, and Z6 (1.43, 0.28 and 0.08 mg/kg, **11**, **12** and **14**, respectively, Figure 3) besides Z2 and Z3, and some of the above cited cytochalasins (*27*).

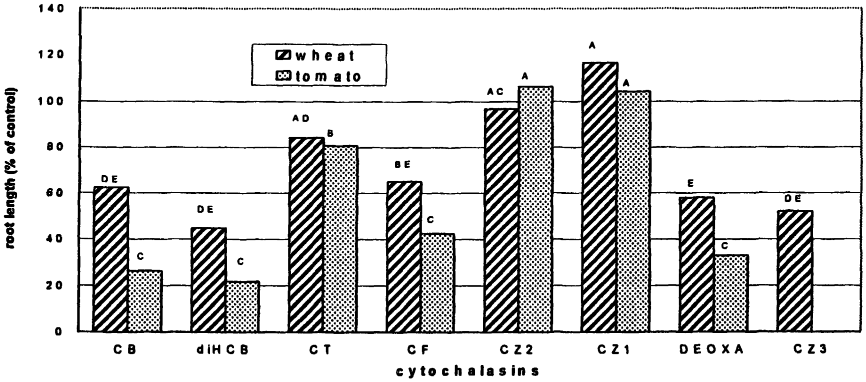


Figure 4. Effect of cytochalasins on root elongation of tomato and wheat seedlings.

Cytochalasins Z4 and Z5 (**11** and **12**) are structurally related to cytochalasin B (**13**), the main toxin produced by both *P. exigua* var. *heteromoprpha* and *P. semeniperda*, as well as other important toxigenic fungi (*24-25*). Together with Z1 (**7**), Z5 (**12**) represents a further example of a 24-oxa[14]cytochalasan bearing a *p*-hydroxybenzyl residue at the C-3 of the perhydroisoindolyl-1-one moiety whereas, in the same group, Z4 (**11**) is the first example of a compound bearing an *o*-hydroxybenzyl residue attached to C-3 (*24-25*). Furthermore, Z6 (**14**) is the first 24-oxa[14]cytochalasan showing at the same time the epoxy group located between C-6 and C-7 of the perhydroisondolyl-1-one residue, the deoxygenation of C-20, and the hydroxylation of C-19 as already observed for Z3 (*27*).

Biological Characterization of Cytochalasins Produced by *P. exigua* var. *heteromorpha*

In the tomato seedling assay, at 10^{-4} M, only Z6 proved to be slightly active causing 30% inhibition of root elongation, whereas Z4 and Z5 were inactive. When assayed at the same concentration on brine shrimp, only Z5 caused a low mortality of larvae (21%), whereas Z4 and Z6 were both inactive (*27*). The results of structure-activity relationship studies (*25-26*) suggest the important role of the hydroxy group at C-7 in conferring the biological activity. This structural feature is present in **11** and **12** (Z4 and Z5) but not in **14** (Z6).

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch005

Therefore, the lack of phytotoxicity in **11** and **12** is probably due to the *ortho*- or *para*-hydroxy substitution of the benzyl residue attached at C-3 suggesting that the presence of an unsubstituted phenyl residue attached at C-10 is important for biological activity (*27*).

Cytochalasins have been considered as potential mycotoxins (*24-25*). If high level of toxins were really produced *in vivo*, this could, in practice, make it hazardous to use *P. semeniperda* as a biological control agent against grass weeds. Hence, studies are in progress both to quantify the presence of such toxins in naturally infected seeds, as well as to estimate their stability and impact in the environment.

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch005

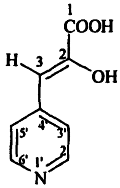
Ascochyta sonchi phytotoxins

Ascochyta sonchi (Sacc.) Grove is a natural pathogen isolated from necrotic leaves of sowthistle (*Sonchus arvensis* L.), a herbaceous weed occurring through the temperate regions of the world. It is being evaluated as a possible biocontrol agent (Berestetski, personal communication). Phytopathogenic fungi belonging to the genus *Ascochyta* are responsible for several diseases that cause necrotic lesions on leaves and stems (*28*). Some *Ascochyta* spp. have also been proposed as mycoherbicides for the biological control of noxious weeds (*4*, *14*). Therefore, the production of toxic metabolites by *A. sonchi*, a promising pathogen, is of interest in view of their use as natural herbicides to be employed in addition to the use of the pathogen or an alternative to it.

Isolation and chemical chracterization of *A. sonchi* phytotoxins

A TLC analysis of the culture filtrates showed the presence of basic metabolites, probably bearing NH_2-groups, and amino acids and/or peptides. Purification of the crude culture filtrate by cationic exchange chromatography resulted in two fractions. The residue obtained by lyophilization of the basic eluate yielded a material containing the main metabolites in almost pure form. The residue, a yellowish highly water-soluble powder having phytotoxic activity, was further purified by a medium pressure silica gel column. The fraction containing the less polar metabolites, which proved to be phytotoxic on leaves, was further purified yielding a homogenous amorphous solid metabolite (1.2 mg/l, **17**, Figure 5), which was named ascosonchine. Ascosonchine, whose structure was determined using essentially spectrocopic methods (IR, 1D and 2D ^{1}H and ^{13}C-NMR and MS), was characterised as (*Z*)-2-hydroxy-3-(4-pyridyl)-2-propenoic acid.

The stereochemistry of the double bond present in ascosonchine was deduced by spectroscopic IR and NMR experiments. The IR absorption bands, in agreement with those reported for the *Z*-enol of phenylpyruvic acid derivatives, allowed assignment of a Z-configuration to the double bond. This *Z*-configuration was further supported by the $^{1}H,^{13}C$ coupling constants recorded in the undecoupled ^{13}C NMR spectrum of **17**. In particular, the value of the vicinal $^{1}H\text{-}C{=}C\text{-}^{13}COOH$ [$^{3}J_{C1\text{-}H3}$=3.7 Hz] is typical for a *cis* arrangement of the coupled nuclei in fragments $^{1}C\text{-}C{=}C\text{-}^{13}C$ with similar sums of electronegative substituents. Presumably, the stability of the *Z*-enol form is due to the conjugation between the phenyl ring, olefinic and carboxyl groups. Therefore, ascosonchine can be formulated as (*Z*)-2-hydroxy-3-(4-pyridyl)-2-propenoic acid (**17**) (*29*).



17

Figure 5. Structure of ascosonchine.

Biological characterization of Ascosonchine

Tested with the leaf puncture assay on the host plant, **17** produced necrotic circular lesions after 2 days resembling those caused by the pathogen. The diameter of the necrotic area appeared very wide (up to 5 mm) when 15 or 3 µg/droplets (around 6 and 1.2 10^{-3} M, respectively) were applied to the leaf surface, and was still quite evident at a concentration five times lower.

Assayed at 15 µg/droplet on several weedy and cultivated plants (monocots and dicots) ascosonchine showed interesting selective properties. In fact, as shown in Table 2, it was completely ineffective on all the solanaceous species assayed (tomato, eggplant, red pepper, potato), was slightly (1-2 mm necrosis) active or almost inactive on leguminous (bean and chickpea) and cucurbitaceous (melon and zucchino) plants, but it caused severe (up to 10 mm) necrosis on many other species, such as *Euphorbia*, *Salvia*, *Valerianella*, or *Triticum*.

Even if further assessments are needed, this semi-selective toxin could have practical applications as a herbicidal compound. It is interesting to note that the toxin is still very active when used at a quite low concentration (*29*).

In the antibiosis assay on *G. candidum*, ascosonchine assayed at concentrations up to 50 µg/disk proved to be completely inactive. The same negative result was observed when the toxin was tested on *Pseudomonas syringae* and *Lactobacillus plantarum* (a Gram- and a Gram+ bacteria, respectively). No effect was observed in the brine shrimp assay as tested at

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch005

concentrations up to 10^{-4} M (*29*). Even if futher nontargeting tests of ascosonchine are required, the results from assays revealed only phytotoxic activity, whereas ascosonchine was completely ineffective on fungi, bacteria and arthropods. This could be very important from a practical point of view, and would confirm the hypothesis that more environmentally friendly and safe herbicides could be obtained by plant pathogenic fungi (*4*).

Table 2. Effect of ascosonchine in the leaf-puncture assay

Common name	*Scientific name*	*Family*	*Effect on leaves*[a]
Alligatorweed	*Alternanthera philoxeroides*	Amaranthaceae	++
Artichoke	*Cynara scolymus*	Compositae	-
Bean	*Phaseolus vulgaris*	Leguminosae	-
Bindweed	*Convolvulus arvensis*	Convolvulaceae	-
Chickpea	*Cicer arietinum*	Leguminosae	+
Eggplant	*Solanum melongena*	Solanaceae	-
Four-o'clock	*Mirabilis jalapa*	Nyctaginaceae	+
Foxtail millet	*Setaria italica*	Poaceae	++
Lamb's lettuce	*Valerianella locusta*	Valerianaceae	+++
Melon	*Cucumis melo*	Cucurbitaceae	-
Pepper	*Capsicum annum*	Solanaceae	-
Potato	*Solanum tuberosum*	Solanaceae	-
Common sage	*Salvia officinalis*	Labiatae	++++
Sowthistle	*Sonchus arvensis*	Asteraceae	+++
Spinach	*Spinacia oleracea*	Chenopodiaceae	+
Sun spurge	*Euphorbia helioscopia*	Euphorbiaceae	++++
Tomato	*Lycopersicon esculentum*	Solanaceae	-
Wheat	*Triticum durum*	Poaceae	+++
Zucchino	*Cucurbita pepo*	Cucurbitaceae	-

[a] Toxicity determined with the following scale: - = no symptoms; + = necrosis with diameter around 1-2 mm; + + = necrosis 2-3 mm; + + + = necrosis 3-5 mm; ++++ = wider necrosis. Results of at least three replicates.

Conclusions

The results obtained recently on the development of weed biocontrol strategies are promising and encouranging, and further experiments are still in progress to overcome the important problems that arise during the practical application of phytotoxins in integrated crop management.

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch005

References

1. Strobel, G. A.; Kenfield, D.; Bunkers, G.; Sugawara, F.; Clardy, J. *Experientia* **1991**, *47*, 819-826.
2. Abbas, H. K.; Duke, S. O. *J. Toxicol.* **1995**, *14*, 523.
3. Duke, S. O.; Dayan, F. E.; Romagni J. G.; Rimando. A. M. *Weed Res.* **2000**, *40*, 9.
4. Evidente, A.; Motta, A. In *Bioactive Compounds from Natural Sources*; Tringali., C., Ed.; Taylor & Francis: London, UK, 2001, Chapter 12, pp 473-525.
5. Evidente, A. *Inf. Fitopatol.* **2002**, *9*, 49.
6. Strobel, G. A. *Ann. Rev. Biochem.* **1982**, *51*, 309.
7. *Phytotoxins and Plant Pathogenesis*; Graniti, A.; Durbin, R.D.; Ballio, A., Eds; Springer-Verlag: Berlin, 1989. NATO ASI Series, Series H, Vol 27.
8. Ballio, A.; Graniti. A. *Experientia* **1991**, *47*, 751.
9. Evidente, A. In *Recent Research Developments in Phytochemistry*; Pandalai, S. G., Ed.; Research Signpost: Trivandrum, India, 1997; pp 255-292.
10. Kempenaar, C.; Horsten, P. J. F. M.; Scheepens P. C. *Eur. J. Plant Pathol.* **1996**, *102*, 143.
11. Holm , L. G.; Pluckett, D. L.; Pancho, J. V.; Herberger, J. P. In *The World's Worst Weed (Distribution and Biology)*; University Press of Haway: Honoloulou, HI, 1977; p 84.
12. Evidente, A.; Capasso, R.; Cutignano, A.; Taglialatela-Scafati, O.; Vurro, M.; Zonno, M. C.; Motta, A. *Phytochemistry* **1998**, *48*, 1131.
13. Evidente, A.; Andolfi, A.; Vurro, M.; Zonno, M. C.; Motta, A. *Phytochemistry* **2000**, *53*, 231.
14. Evidente, A.; Andolfi, A.; Vurro, M.; Zonno, M. C. *Phytochem. Anal.* **2001**, *12*, 383.
15. Bassarello, C.; Bifulco, G.; Evidente, A.; Riccio, R.; Gomez-Paloma, L. *Tetrahedron Lett.* **2001**, *42*, 8611.
16. Andreatta, R. H.; Nair, V.; Robertson, A. V.; Simpson, W. R. J. *Austral. J. Chem.* **1967**, *20*, 1493.
17. Vurro, M.; Zonno, M. C.; Evidente, A.; Andolfi, A.; Montemurro, P. *Biol. Control* **2001**, *21*, 182.
18. Netland, J.; Dutton, L. C.; Greaves, M. P.; Baldwin, M.; Vurro, M.; Evidente, A.; Einhorn, G.; Scheepens, P. C.; French, L. W. *BioControl* **2001**, *46*, 175.
19. Campbell, M.A.; Medd, R.W.; Brown, J.F. In *Proceedings of the 9th International Symposium on Biological Control of Weeds*; Stellenbosch, South Africa, 1996; pp 519-523.
20. Medd, R.H. *Rev. Plant Pathol.* **1992**, *71*, 891.
21. Neergard, P. *Seed Pathology*; The Macmillan Press: London, 1979, Vol. 1.

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch005

22. Bach, E.; Christiansen, S.; Dalgaard, L.; Larse, P. O.; Olsen, P. E.; Smedergard-Peterson, V. *Physiol. Plant Pathol.* **1979**, *14*, 41.
23. Friis, P.; Olsen, C.E.; Moller, B.G. *J. Biol. Chem.* **1991**, *266*, 13329.
24. Natori, S.; Yahara, I. In *Mycotoxins and Phytoalexins*; Sharma, R. P.; Salunke, D. K., Eds.; CRC Press: Boca Raton, FL, 1991; Chapter 12, pp 291-336.
25. Vurro, M.; Bottalico, A.; Capasso, R.; Evidente, A. In *Toxins in Plant Disease Development and Evolving Biotechnology*; Upadhyay, R. K.; Mukerji, K. G., Eds; Oxford & IBH Publishing Co. PVT. LTD: New Delhi, India; 1997, pp 127-147.
26. Evidente, A.; Andolfi, A.; Vurro, M.; Zonno, M. C.; Motta, A. *Phytochemistry* **2002**, *60*, 45.
27. Evidente, A.; Andolfi, A.; Vurro, M.; Zonno, M.C.; Motta, A. *J. Nat. Prod.* **2003**, *66*, 1540.
28. Melnik, V. A. *Mikologia i Fitopatologia* **1971**, *5*, 15.
29. Evidente A.; Andolfi, A.; Abouzeid, M.A.; Vurro, M. *Phytochemistry* **2004**, *65*, 475 (and references therein cited).

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch005

Chapter 6

Natural Compounds for Novel Strategies of Parasitic Plant Management

Maurizio Vurro and Angela Boari

Institute of Sciences of Food Production, CNR, Via Amendola 122/O, 70125 Bari, Italy

Downloaded by CORNELL UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch006

Parasitic plants are among the worst weed problems, being responsible for major losses to many crops. Considering that seed germination is one of the key phases for parasite development, natural compounds could play interesting roles in the management of parasitic plants. For example, fungal toxins produced by phytopathogenic fungi could be used, depending on their biological and chemical characteristics, as natural herbicides or as biomarkers to select more efficacious mycoherbicides. Alternative strategies, such as the use of natural amino acids to cause physiological disorders of germinating seeds, the selection of more virulent fungi producing toxins, and the use of metabolites isolated from host root exudates for soil application, to stimulate "suicidal germination" of seeds and reduce the seed bank, are also possible.

The Problem

Species of the genus *Orobanche*, commonly called broomrapes, are among the worst parasitic weed species of the world, being responsible for severe losses to vegetable, legume and sunflower crops in Europe, including the Balkans and Russia, the Middle East and North Africa (*1*), by interfering with water and mineral intake and by affecting photosynthate partitioning.

In Natural Products for Pest Management; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

Downloaded by CORNELL UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch006

Difficulties in broomrape control are due to the large amount of seeds produced, that can remain viable for many years. They germinate only if stimulated by host root exudates, and produce a germ tube that, if it attaches to the host root, develops a haustorium penetrating the root and forming a tubercle. This is followed by the most damaging phase, a long underground one, during which the parasite withdraws water, nutrients and photosynthates from the host. Parasite emergence occurs only when most of the damage has already been produced.

Traditional control methods have been tried on different crops, but none has proved to be effective. *Orobanche* spp. cannot usually be managed by persistent selective herbicides, since herbicides are not able to differentiate between the crop and the parasite, except on herbicide-tolerant transgenic crops (*2*). Multiple applications of low rates of crop-degraded herbicides can provide a modicum of control and may be more useful when integrated with other methods (*3*). Seed eradication by solarization or soil fumigation is effective but expensive, and soil sterilization by fumigation with methyl bromide and ethylene dibromide is mostly banned due to environmental risks (*4*). Furthermore, mechanical control is impossible, except by removing flower stalks to reduce seed production and dispersal, because these weeds attach to crop roots. Although many promising agents have been isolated, no pathogenic organisms have been developed as mycoherbicides for *Orobanche* biocontrol (*5, 6*).

Considering that the germination of seeds is a key phase of the parasitic plant life cycle, searching for natural compounds able to inhibit seed germination or to stimulate it in absence of the host, consequently reducing the seed bank, seems to be an attractive and environmentally friendly approach.

Use of natural compounds as herbicides

Fungi represent an enormous source of metabolites, mostly still unexplored. Toxic metabolites produced by fungal pathogens can: belong to different chemical families (i.e.: glycosides, peptides, phenolics, terpenoids); have different ecological and environmental roles, such as to be important factors for pathogenicity or virulence; have different behaviors with respect to the host, varying from strictly host-specific to completely non-specific compounds; and act with different mechanisms, affecting several sites in the host.

Fungal species belonging to the same genus are able to produce a wide variety of metabolites, e.g.: *Alternaria, Claviceps* or *Fusarium* species, for which more than one hundred of secondary metabolites have been isolated. A further source of variability is that toxins belonging to the same structural group can be produced by different microorganisms belonging to many different genera. This is the case, for example, of cytochalasins, produced by more than 30 different fungal species (*7*); or trichothecenes, a group including more than 100 different compounds, produced by different genera, such as *Fusarium* (more than 25 different trichothecenes), *Myrothecium* (producing roridins and verrucarins), and *Trichoderma* (trichodermins) (*8*); or destruxins, metabolites known for their herbicidal and insecticidal properties, produced in tens of different forms (*9*).

Phytotoxins from fungal pathogens of crops have received considerable attention mainly in the understanding of disease development and in setting up strategies for disease control, but much less attention has been given to the secondary metabolites produced by weed pathogens. Usually bioactive compounds produced by plant pathogenic fungi, including those attacking weeds, are considered risky compounds, because they have been intensively studied mainly in relation to the negative effects to human and animal health when these toxins accumulate in agricultural commodities and are absorbed through nourishment. Besides a few families of metabolites, such as ochratoxins, aflatoxins, trichothecenes, zearalenols, fumonisins and alkaloids of *Claviceps*, which have proved to be responsible for severe human and animal poisonings, there are many other metabolites that are not so dangerous, and some of them have been proposed to be used as natural herbicides (*10, 11*).

With regard to infesting broomrapes, Zonno and Vurro (*12*) have recently shown that some toxins produced by fungi of the genus *Fusarium* were able to inhibit germination of *O. ramosa* seeds, and proposed their practical use for parasitic plant management. An assay on stimulated germinating seeds was used. Briefly, *O. ramosa* seeds are sterilized in sodium hypochlorite. Seeds are then rinsed with sterile tap water and conditioned on wet glass microfibre filters in Petri dishes, at 26° C in the dark for 3 weeks. Filters are then cut in small pieces, each containing around 100 seeds. The pieces are placed on another filter moistened with 2 ml of the assay solution, containing the toxin and GR24 (a synthetic stimulant) and kept at 25° C in the dark. After 4 days, the percentage of seed germination is determined and compared with that of the control, prepared with the same procedure as the treatments but without the toxin.

Eighteen toxins were assayed, and seven of them (diacetoxyscirpenol, HT-2 toxin, neosolaniol, T-2 toxin, fusarenon X, deoxynivalenol, nivalenol) caused 100% inhibition of germination when assayed at 10^{-5} M.

All the other toxins were active at different levels when assayed at 10^{-4} M, ranging between 63 % of germination (moniliformin) to almost inactive (around 92% of germination) in case of enniatin. The strongest toxins at 10^{-6} M, T-2,

Downloaded by CORNELL UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch006

HT-2, neosolaniol and diacetoxyscirpenol, caused an almost complete inhibition of seed germination (*12*).

Some of those toxins are powerful mammalian mycotoxins, and the risk of introducing mammalian toxic compounds into the environment should be carefully ascertained. All four most active compounds belong to the trichothecene A group (Figure 1), a well known group of mammalian mycotoxins (*13, 14*); the other three most active compounds belong to the trichothecene B group (Figure 2).

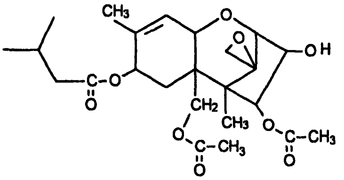


Figure 1. Chemical structure of T-2 toxin, belonging to the trichothecene A group.

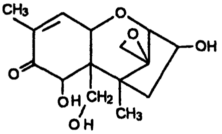


Figure 2. Chemical structure of deoxynivalenol, belonging to the thricothecene B group.

Thus, the common chemical structure of those compounds seems to play an important role in blocking the germination of stimulated seeds. The trichothecenes are a family of tetracyclic sesquiterpenoid substances produced by several species of *Fusarium*. More than 100 compounds are known. They cause a wide variety of biological effects owing to the diversity of chemical structures within the group. They all are potent inhibitors of protein synthesis in eucaryotic cells (*15*).

The results are in agreement with a previous study, using 14 fungal toxins on germination of seeds of *Striga hermonthica*, a hemiparasitic weed infesting cereals (*16*). In fact, T-2 and deoxynivalenol were also the most active, at very low concentrations against *Striga*. Enniatin and beuvericin were slightly more active against *Striga*, whereas nivalenol was much stronger against *Orobanche*. The activities of fusaric and dehydrofusaric acid, as well as of their respective methyl esters, were much stronger against *Striga* than against *Orobanche*.

More recently, many fungi were isolated from diseased *O. ramosa* plants during extensive field surveys carried out in Southern Italy, and some of them proved to be promising potential mycoherbicides for biological control of

Downloaded by CORNELL UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch006

broomrapes (*6*). Fifty-three isolates tested for virulence were also grown *in vitro* both on liquid and solid media with the main aim to find new metabolites having the ability to inhibit the induced germination of *O. ramosa* seeds (*17*).

Among them, one strain of *Myrothecium verrucaria* and one of *Fusarium compactum* were able to produce some metabolites very active in inhibiting the germination of *O. ramosa* seeds, when grown in liquid culture. Eight metabolites were isolated from *M. verrucaria* culture extracts. Seven of them, identified by spectroscopic methods, proved to be macrocyclic trichothecenes, namely verrucarins A, B, M and L acetate, roridin A, isotrichoverrin B and trichoverrol B (Figure 3).

The main metabolite was identified as verrucarin E (Figure 4), a disubstituted pyrrole not belonging to the trichothecene group. The main metabolite produced by *F. campactum* was identified as neosoloaniol monoacetate (Figure 4), a further well known trichothecene (*18*).

Verrucarins A, B, M and L acetate belong to a subgroup of macrocyclic trichothecenes having a differently functionalized lactone ring located between C-4 and C-15. This macrocycle was substantially different and open, respectively, in roridin A, isotrichoverrin B and trichoverrol B, which belong to two other subgroups of the macrocyclic trichothecene family (*19, 20, 21*).

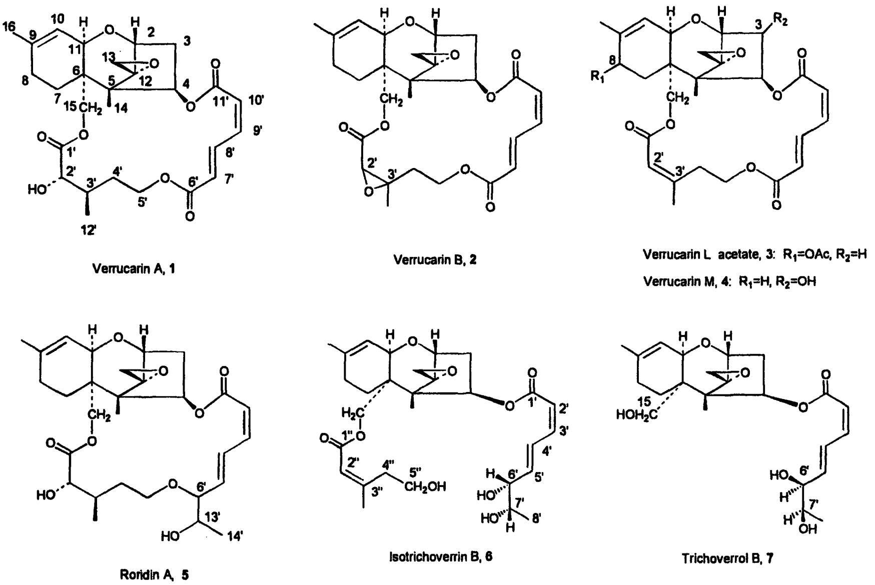
Assayed on *O. ramosa* seeds at 10^{-4} M, all the metabolites caused the total inhibition of the stimulated germination, except verrucarin E, not belonging to the trichothecene group, which proved to be inactive. At 10^{-5} M all the trichothecenes were still highly active, causing total inhibition of seed germination, except isotrichoverrin B, which was slightly less toxic. At a concentration 10 times lower, most of the metabolites were still active, except verrucarin M, which proved to be almost inactive, besides isotrichoverrin B and verrucarin E. Particularly noteworthy is the activity, at this concentration, of roridin A and neosolaniol monoacetate, both able to cause 100% inhibition of seed germination (*18*).

Although the evaluation of the *in vitro* activity of hazardous metabolites appears to be important, and could also represent a preliminary assessment of the risk due to the introduction of mycoherbicides into the environment, the "real" fate of those metabolites when applied in the environment should be carefully ascertained, both to prevent contamination that would have a significant impact on the environment and consequently a negative impact on public perceptions, but also to prevent discarding useful compounds or the producer microorganisms because of concerns about their use. For example, neither intact macrocyclic trichothecenes nor toxic metabolites in plant tissues after treatment of kudzu (*Pueraria montana*) with the proposed mycoherbicide *M. verrucaria* could be detected (*22*).

Considering the efficacy of some toxins at very low concentrations, the possibility of using fungal toxins as natural herbicides to inhibit germination of

Downloaded by CORNELL UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch006

parasitic plant seeds seems to be not so remote. Many toxins are not selective, being able to cause the same toxic effects both on host as well as on non-host plants. For this reason, the toxicity to crop plants has to be ascertained, even if the application is at very low concentration and their quick degradation after inhibition of seed germination could avoid toxic effects. Fungal culture extracts could be an interesting source of new compounds acting as natural and original germination inhibitors.


Verrucarin A, 1

Verrucarin B, 2

Verrucarin L acetate, 3: R_1=OAc, R_2=H

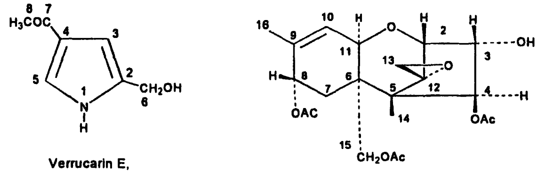
Verrucarin M, 4: R_1=H, R_2=OH

Roridin A, 5

Isotrichoverrin B, 6

Trichoverrol B, 7

Figure 3. Chemical structure of macrocyclic trichothecenes.


Verrucarin E,

Neosolaniol monoacetate

Figure 4. Chemical structure of verrucarin E and neosolaniol monoacetate.

From a practical point of view, considering that most of the crop plants parasitized by broomrape are irrigated, toxins could be introduced by drip irrigation systems, in very low amounts near the host roots. This could prevent the germination of the seeds only where needed, preventing attachment of

Downloaded by CORNELL UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch006

haustorium to the host roots. This should minimize environmental risks, reducing the amount of toxins to be applied and avoiding toxin dispersal. Furthermore, being natural products, those metabolites should be easily bio-inactivated soon after their application, reducing risks of drifting and dispersal. In any case, it is of utmost importance to evaluate the fate of the metabolites, including the assessment of the stability and their movement into the soil, in order to consider the real risk of their introduction in the environment.

Use as biomarkers

A different potential use of toxic metabolites produced by weed pathogens is as "biomarkers". If toxins proved to be virulence factors, meaning there is a positive correlation between toxin production and aggressiveness of the candidate mycoherbicide, more virulent strains of the pathogens could be selected simply by testing the production *in vitro* of toxic metabolites and choosing the highest toxin producers.

In this regard 53 strains, isolated from diseased *O. ramosa* plants during extensive field surveys carried out in Southern Italy, were grown in both in liquid and solid media (*17*). The ability of the extracts to inhibit seed germination was evaluated, and the production *in vitro* of fusaric and dehydrofusaric acids (FA and DFA, respectively) by *Fusarium* strains was estimated. The possible correlation of toxin content and toxicity of extracts with virulence of strains was ascertained.

Almost all the strains that proved to be highly virulent were also able to produce liquid cultures with a very high efficacy in the *O. ramosa* seed germination assay. Only two of the tested strains (one of *F. camptoceras* and one of *F. oxysporum*) were highly virulent without any production of bioactive metabolites from either liquid or solid cultures. Conversely, only one fungus, a strain of *M. verrucaria* appeared to be a very interesting source of toxic compounds, although it proved to be only weakly virulent. Some other strains were neither virulent nor producers of bioactive metabolites, and it could be they were saprophytes occasionally isolated from *Orobanche* tissues.

In that study, no correlation was found between the content of FA and DFA and the toxicity of the extracts of liquid cultures, with the exception of a strain of *Fusarium* sp. whose liquid extract had both high contents of DFA (204 mg/l) and FA (76 mg/l), as well as high toxicity. In fact, the extract of a strain of *F. oxysporum*, which had one of the highest contents of FA (more than 166 mg/l), was inactive on seed germination, whereas that of another strain of *F. oxysporum*, with one of the lowest FA contents (29 mg/l), caused about 66% inhibition of seed germination. The same was true for DFA. In fact, the extract of one strain of *F. oxysporum*, having one of the highest contents (more than 195

Downloaded by CORNELL UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch006

mg/l), caused 31% inhibition, around the same caused by a strain of *F. proliferatum*, one of the weakest DFA producers (around 17 mg/l) (*17*).

Moreover, among *F. oxysporum* strains, comparison of the production of metabolites with the virulence of the strains did not show a positive correlation. Two strains that were among the best FA and DFA producers caused no disease. Conversely, another strain, that produced quite low amounts of toxins, proved to be one of the strongest pathogens of *O. ramosa* (*17*).

Use of amino acids

Downloaded by CORNELL UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch006

This approach is modeled on the mechanism of action of the so called "Frenching disease", a disease of tobacco caused by saprophytic bacteria growing on the roots, overproducing isoleucine (*23*). The end products of the branched chain amino acid pathway (valine, leucine, and isoleucine) allosterically regulate the activity of acetolactate synthase (ALS). The isoleucine overproduction inhibited the activity of ALS in the tobacco, shutting down synthesis of valine and leucine, which disrupted essential protein metabolism. Several modern chemical herbicides also inhibit single biosynthetic enzymes in plants (including ALS), rendering treated plants incapable of producing a metabolite essential for plant growth. The idea, in this case, is to supply high or unbalanced amounts of essential amino acids to cause disorders in the physiological processes that occur during the germination of seeds, inhibiting the process of germination or that of germ tube elongation. Some preliminary experiments carried out using the biological assay described above showed the ability of some amino acids to inhibit germination of stimulated parasitic seeds. In particular, in the case of *O. ramosa,* the most interesting results were obtained supplying proline, arginine, histidine and methionine, whereas for *Striga hermonthica*, a parasitic weed attacking mainly cereals, encouraging preliminary results were obtained using leucine, threonine and tyrosine (*24*). In particular, methionine was able to inhibit germination of *O. ramosa* seeds if applied within three days after the addition of the stimulant to the seeds, whereas its inhibitory activity was overcome by seed washing. Furthermore, both the length and the shape of the germinated seeds treated with different concentrations of methionine seemed to be influenced by the concentration of the amino acid supplied (*24*).

Amino acids are not normally toxic to humans or to the environment, and are rapidly metabolized by soil microorganisms, so that they could be "safe" method of parasitic weed management. Studies, carried out by the authors in collaboration with Prof. David C. Sands, Montana State University, Bozeman, USA, are in progress to evaluate the possible use of this interesting approach to inhibit the germination of parasitic weed seeds and to understand the physiological mechanisms of action.

Obtaining more virulent mycoherbicides due to toxin overexpression

The development of pathogens with enhanced biocontrol activity by selection or by the introduction of genes responsible for toxin or other metabolite biosynthesis seems a reasonable possibility. Several genes in the biosynthetic pathways of fungal toxins have already been identified and cloned, such as many of those encoding the biosynthetic pathway of trichothecenes (*25 and ref. therein cited*) and toxins produced by several species of different fungal genera such as *Fusarium* and *Trichoderma*.

The protein NEP1 is a potent phytotoxin isolated for the first time from culture filtrates of a strain of *Fusarium oxysporum* pathogenic for *Erythroxylum coca*, and later found as a product of many other strains of the same species (*26*). The toxin, inducing necrosis in leaves, is responsible for the natural virulence of the species producing it, and the gene *nep1*, encoding that proteinaceous toxin, has been identified. Recently, Amsellem *et al.* (*27*) have transferred the *nep1* gene to a weak pathogenic strain of *Colletotrichum coccodes*, a potential mycoherbicide for the biological control of *Abutilon theophrasti*. Compared to the wild type, the obtained transgenic strain was more virulent, faster in causing the disease, and with a reduced requirement for the dew period.

With regard to the possibility of obtaining mycoherbicides overproducing amino acids, variants of *F. oxysporum* f. sp. *cannabis* resistant to valine analogs were obtained (*23*). These resistant variants excreted 10-55 times more valine than their wild type parent, and proved to be more virulent to *Cannabis sativa* than the wild type parent. The wild type strain resulted in 25% control of the target plant, while the valine mutants increased control to 70-90%. In addition, the development of wilt disease was more rapid in the plants infested with the valine overproducers. Thus, overproduction of an essential amino acid could provide a highly effective means of enhancing the virulence of a biocontrol agent (*23*).

Use of seed germination stimulants

Considering that the germination of seeds of parasitic plants depends on chemicals exuded from the roots of the host plant, an alternative approach for the management of parasitic weeds could be the reduction of the amount of parasite

Downloaded by CORNELL UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch006

seeds in the soil by "suicidal" germination. This can be obtained by the application of a germination stimulant to the soil, in absence of the host. The parasite seeds will germinate but, in absence of the host, they will die.

The chemical nature of such germination stimulants is well known in the case of *Striga* spp. (*28*), whereas less information is available for other parasitic plants. The *Striga* spp. germination stimulants reported, isolated from both host and non-host plants, belong to different chemical groups, most of them being sesquiterpenes. Strigol is the most representative compound of the largest group, named strigolactones (*28*) and was the first germination stimulant characterized (*29*). Two stimulants of *O. minor* seeds, alectrol and orobanchol, have been isolated from the root exudates of *Trifolium pratense*, the host plant (*30*). More recently, six sunflower sesquiterpene lactones were tested as *O. cumana* germination stimulants, and two of them significantly increased seed germination. The effect of these two compounds is species-specific, showing no germination stimulant activity on other *Orobanche* spp. tested (*31*).

Strigol has been the starting point for the development of the synthetic GR family, among which GR24 is the commonest compound used for lab studies. More recently, another synthetic stimulant, named Nijmegen 1, has been developed. This compound can be more easily synthesized compared to other compounds and, because of its biological activity in germinating parasitic weed seeds and its easier preparation, it has been proposed for a practical use in the field (*32*).

Considering that large amounts of stimulants should be available for their practical application at a field level, limiting factors for their marketing and use are: long, difficult and highly costly synthesis processes, in the case of synthetic compounds; and difficulties to obtain large amounts and high cost of purification, in the case of natural compounds. Alternative strategies to obtain low-cost natural stimulants could be, for example, the use of metabolites stimulating the suicidal germination produced by fungi (*33*), suitable for mass production, or the purification from residual nutrient solutions obtained by NFT (Nutritive Film Technique) tomato crops (*24*).

Downloaded by CORNELL UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch006

Conclusions

The use of natural compounds could offer more environmentally friendly and more efficacious approaches for the management of parasitic weeds. Natural metabolites could be used directly, as natural herbicides, and indirectly, as biomarkers. Alternative strategies, such us the use of metabolites isolated from host root exudates and from fungal cultures for soil application to stimulate the "suicidal germination" of seeds and reduce the seed bank, or of natural amino acids to cause physiological disorders, is also possible. Concerted scientific efforts are needed to develop these strategies.

References

1. Sauerborn, J. Fifth International Symposium on Parasitic Weeds, Nairobi, Kenya, June 24th-30th 1991, pp. 137-143.
2. Aviv, D.; Amsellem, Z.; Gressel, J. *Plant Sci.* **2002**, *58*, 1187-1193.
3. Hershenhorn, J.; Goldwasser, Y.; Plakhine, D.; Ali, R.; Blumenfeld, T.; Bucsbaum, H.; Herzlinger, G.; Golan, S.; Chilf, T.; Einzenberg, H.; Dor, E.; Kleifeld, Y. *Weed Res.* **1998**, *38*, 343-349.
4. Foy, C. L.; Jain, R.; Jacobsohn, R. *Rev. Weed Sci.* **1989**, *4*, 123-52.
5. Amsellem, Z.; Kleifeld, Y.; Kerenyi, Z.; Hornok, L.; Goldwasser, Y.; Gressel, J. *Biol. Control* **2001**, *21*, 274-284.
6. Boari, A.; Vurro, M. *Biol. Control* **2004**, *30*, 212-219.
7. Vurro, M.; Bottalico, A.; Capasso, R.; Evidente, A. In *Toxins in Plant Disease Development and Evolving Biotechnology*; Upadhyay R. K.; Mukherji, K. G., Eds.; Oxford & IBH Publishing Co. Ltd: New Delhi, India, 1997; Chapter 7, pp. 127-147.
8. Lacey, J. *Trichothecenes and other mycotoxins*; John Wiley and Sons: Chichester, UK, 1985.
9. Pedras, M. S. C.; Zaharia, L. I.; Ward, D. E. *Phytochemistry* **2002**, *59*, 579-596.
10. Strobel, G. A.; Kenfield, D.; Bunkers, G.; Sugawara, F.; Clardy, J. *Experientia* **1991**, *47*, 819-826.
11. Evidente, A.; Motta, A. In *Bioactive Compounds from Natural Sources*; Tringali, C., Ed.;: Taylor & Francis: London, UK, 2001; p. 473-525.
12. Zonno, M. C.; Vurro, M. *Phytoparasitica* **2002**, *30*, 519-524.
13. Cheeke, P.R. Natural Toxicant in Feeds, Forages, and Poisonous Plants. Interstate Publishers Inc.: Danville, IL, 1998.
14. Ueno Y. *Trichothecenes. Chemical Biological and Toxicological Aspects.* Elsevier: Amsterdam, Netherlands, 1983.
15. Feinberg, B.; McLaughlin, C. S. In *Trichothecene Mycotoxicosis: Pathophysiologic Effects*; Beasley, V.R., Ed.; CRC Press: Boca Raton, FL, 1989; Vol. 1, pp 27.
16. Zonno, M. C.; Vurro, M. *Weed Res.* **1999**, *39*, 15-20.
17. Abouzeid, M. A.; Boari, A.; Zonno, M. C.; Vurro, M.; Evidente, A. *Weed Sci.* **2004**, *52*, 326-332.
18. Andolfi, A.; Boari, A.; Evidente, A.; Vurro, M. *J. Agric. Food Chem.* **2005** *53*, 1598-1603.

Downloaded by CORNELL UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch006

19. Cole, J. C.; Cox, R. H. *Handbook of Toxic Fungal Metabolite.* Academic Press: New York, US, 1981.
20. Jarvis, B. B.; Stahly, G. P.; Pavanasasivam, G.; Midiwo, J. O.; DeSilva, T.; Holmlund, C. E. *J. Org. Chem.* **1982**, *47*, 1117-1124.
21. Jarvis, B. B.; Wang, S.; Ammon, H. L. *J. Nat. Prod.* **1996**, *59*, 254-261.
22. Abbas H. K.; Tak, H.; Boyette, C. D.; Shier, W. T.; Jarvis, B. B. *Phytochemistry* **2001**, *58*, 269-276.
23. Sands D. C.; Tiourebaev, K. S.; Pilgeram, A. L. In *Enhancing Biocontrol Agents and Handling Risks. NATO Science Series: Life and Behavioral Sciences*, Vurro M., et al. Eds.; IOS Press: Amsterdam, Netherlands, 2001; Vol. 339.
24. Boari, A. Personal communication.
25. Proctor, R. H.; Desjardins, A. E.; McCormick, S. P.; Plattner, R. D.; Alexander, N. J.; Brown, D. W. *Eur. J. Plant Pathol.* **2002**, *108*, 691-698.
26. Bailey, B. A.; Jennings, J. C.; Anderson, J. D. *Can. J. Microbiol.* **1997**, *43*, 45-55.
27. Amsellem, Z.; Cohen, B.; Gressel, J. *Nature Biotech.* **2002**, *20*, 1035-1039.
28. Butler, L. G. In *Allelopathy: Organisms, Processes and Applications,* Inderjit, K. M.; Dakshini, M.; Einhellig, F. A., Eds.; ACS Symposium Series, American Chemical Society, Washington, DC, 1995, pp. 158-168.
29. Cook, C. E.; Whichard, L. P.; Wall, M. E.; Egley, G. H.; Coggon, P.; Luhan, P. A.; McPhail, A. T. *J. Am. Chem. Soc.* **1972**, *94*, 6198-6199.
30. Yokota, T.; Sakai, H.; Okuno, K.; Yoneyama, K.; Takeuchi, Y. *Phytochemistry* **1998**, *40*, 1967-1973.
31. de Luque A. P.; Galindo, J. C. G.; Macias, F. A.; Jorrin, J. *Phytochemistry* **2000**, *53*, 45-50.
32. Nefkens, G. H. L.; Thuring, J. W. J. F.; Beenakkers, M. F. M.; Zwanenburg, B. *J. Agric. Food Chem.* **1997**, *45*, 2273-2277.
33. Yoneyama, K.; Takeuchi, Y.; Ogasawara, M.; Konnai, M.; Sugimoto, Y.; Sassa, T. *J. Agric. Food Chem.* **1998**, *46*, 1583-1586.

Downloaded by CORNELL UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch006

Downloaded by COLUMBIA UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch007

Chapter 7

Search for Germination Stimulants and Inhibitors for Root Parasitic Weeds

Koichi Yoneyama[1], Daisuke Sato[1], Yasutomo Takeuchi[1], Hitoshi Sekimoto[2], Takao Yokota[3], and Takashi Sassa[4]

[1]Center for Research on Wild Plants and [2]Faculty of Agriculture, Utsunomiya University, Utsunomiya 321-8505, Japan
[3]Department of Biosciences, Teikyo University, Utsunomiya 320–8511, Japan
[4]Faculty of Agriculture, Yamagata University, Tsuruoka 997–8555, Japan

Germination of root parasitic weeds *Striga* and *Orobanche* is induced only after an exposure to stimulants in root exudates of host and some non-host plants. Since the isolation and characterization of strigol as the first *Striga* germination stimulant, five strigol-related compounds, termed strigolactones, have been isolated and characterized as natural stimulants. However, plants seem to produce many other stimulants including novel strigolactones. In addition to these natural stimulants, fungal metabolites, cotylenins and fuscicoccins, and plant hormone jasmonate and its analogues were found to elicit germination of these root parasites. In this paper, characterization and structure-activity relationships of these germination stimulants and other fungal metabolites as germination inhibitors are discussed.

Among the parasitic angiosperms, witchweeds (*Striga* spp.) and broomrapes (*Orobanche* spp.) are the two most devastating weeds on several cereal and leguminous crops, respectively. These parasites produce large numbers of tiny seeds with prolonged viability and special germination requirements. To germinate, the seeds should be kept in a warm moist environment for several days (termed conditioning) and subsequently be exposed to exogenous germination stimulants (*1*). Once germinated, these parasites will die within a week unless they attach to the roots of host plants. Therefore, inducing seed germination in the absence of host plants, termed "suicidal germination", is a promising strategy for depleting seed reserves in soil (*2*). For this purpose, germination stimulants that are stable under field conditions should be found or developed, because natural germination stimulants identified so far are rather unstable and decompose rapidly in soil. On the other hand, compounds that inhibit germination of these parasites without causing damage to host plants and to the environment are of practical importance, since those compounds may be used in the heavily-infested fields where crops susceptible to the parasites are to be planted. In this report, occurrence and structures of natural and false germination stimulants for root parasites, *Striga* and *Orobanche*, are presented along with inhibitors of germination.

Downloaded by COLUMBIA UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch007

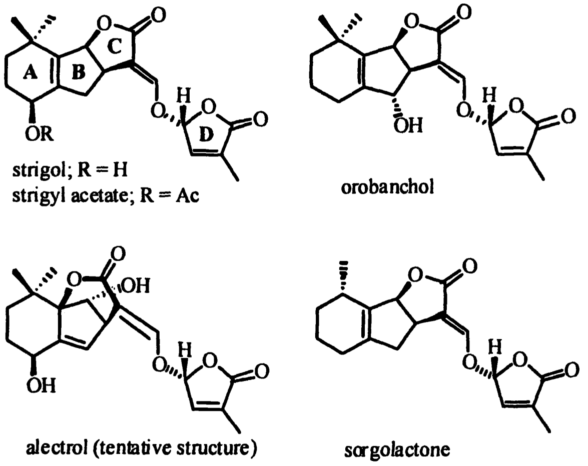
Germination Stimulants

It should be emphasized that conditioned seeds of root parasites *Striga* and *Orobanche* will not germinate unless they are exposed to exogenous germination stimulants. There are some reports on "spontaneous germination" in the absence of such a stimulant, but most of them were not reproducible. It is likely that, under natural conditions, a portion of parasite seed population in soil are conditioned and only a portion of them receive germination stimulants. Since some of these root parasites parasitize a wide range of plant species, many plant species produce and release germination stimulants for these parasites. In addition, as discussed later, some microorganisms were found to produce chemicals that elicit parasite seed germination as well as other chemicals that inhibit germination. Therefore, germination rates of these parasites in soil might be relatively high.

In this paper, these germination stimulants are classified into two groups; natural germination stimulants which play important roles in host recognition by root parasites and false germination stimulants that are not involved parasite-host interactions and thus may be used as suicidal germination stimulants.

Natural Germination Stimulants; Strigolactones

A group of sesquiterpene lactones, collectively called strigolactones (Figure 1) are potent germination stimulants for both *Striga* and *Orobanche*. To date, 5 natural strigolactones have been characterized. They are strigol, strigyl acetate, sorgolactone, alectrol (tentative structure), and orobanchol. Strigol and strigyl acetate, the first strigolactones, were isolated from root exudates of a false host cotton (*3, 4*). Later, Siame et al. identified strigol in the root exudates of genuine hosts of *Striga*, sorghum, maize, and proso millet (*5*). Therefore, strigol is produced by both host and non-host plants. By contrast, all of the other natural strigolactones have been isolated from host plants; sorgolactone from sorghum (*6*), alectrol from cowpea (*7*), and orobanchol from red clover (*8*). It is likely, however, that these strigolactones are also produced by non-host plants.



strigol; R = H
strigyl acetate; R = Ac

orobanchol

alectrol (tentative structure)

sorgolactone

Figure 1. Chemical structures of natural strigolactones.

In the search for natural germination stimulants, various hosts of *Striga* or *Orobanche* were grown hydroponically and root exudates were collected weekly. These root exudates were extracted with ethyl acetate, and the ethyl acetate extracts were examined for germination stimulation activity on *O. minor* Sm. The results are summarized in Figure 2 in which germination rates of the seeds treated with a portion of the ethyl acetate extracts corresponding to 1 mL, 5 mL or 20 mL of the root exudates are shown. In Figure 2, the number of plants, their growth stages, and growth media were optimized to each plant species, and thus direct comparison of germination stimulation activity between different plant species is not applicable. Most of plant species examined were found to produce

Downloaded by COLUMBIA UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch007

germination stimulants immediately after germination. However, for example, the root exudates of young tomato seedlings (~3 week-old), a host of *Orobanche*, did not induce germination at all. Furthermore, nutrients influenced production of germination stimulants and their effects varied with plant species.

Since most of the plants grown hydroponically produced germination stimulants, crude extracts of root exudates were purified by reverse phase (ODS) HPLC. The fractions obtained were tested for germination stimulation on *O. minor.*

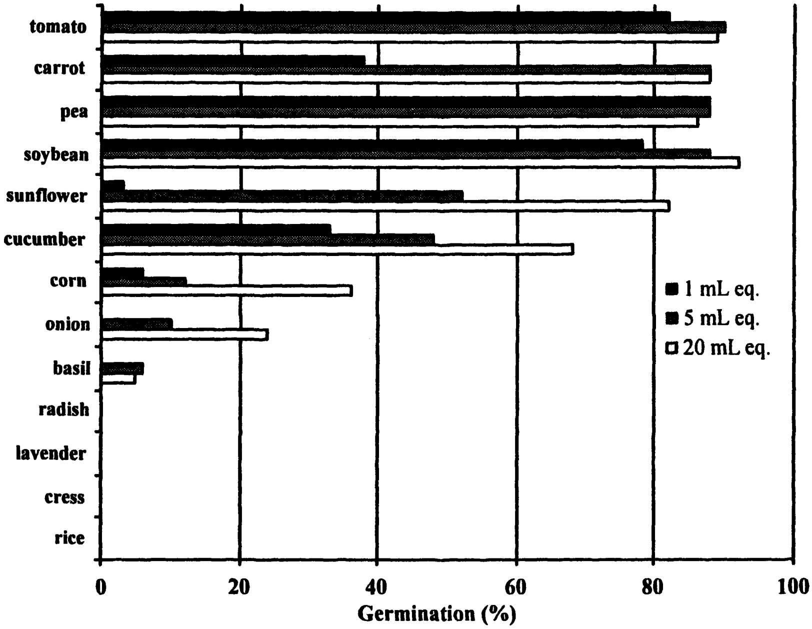


Figure 2. Germination stimulation on Orobanche minor *by root exudates of various crop plants.*

In the case of soybean, for example, there were two active fractions after HPLC separation of ethyl acetate extracts of root exudates and these fractions seemed to correspond to orobanchol and alectrol based on their retention times. The same sample was then analyzed by HPLC-tandem mass spectrometry (LC/MS/MS) using multiple reaction monitoring (MRM) method. For MRM, transition of *m/z* 369 > 272 corresponding to $[M + Na]^+$ and $[M + Na - D\ ring]^+$, respectively, was monitored to detect strigol and its isomers including orobanchol and alectrol (*9, 10*). In the MRM chromatograms shown in Figure 3

Downloaded by COLUMBIA UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch007

Downloaded by COLUMBIA UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch007

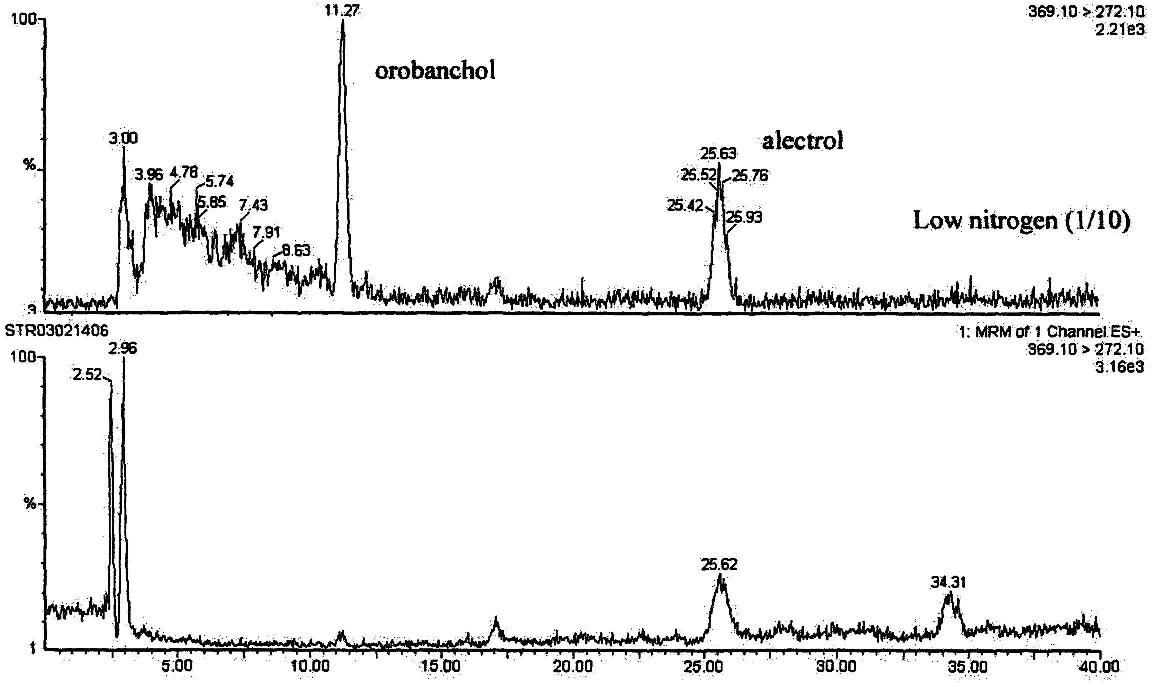


Figure 3. MRM chromatograms of soybean root exudates grown under low nitrogen (upper, 1/10 N) and normal conditions (lower).

(clear in the upper chromatogram), two peaks were detected at retention times of 11.3 and 25.6 min, corresponding to orobanchol and alectrol, respectively.

The effect of nutrients (nitrogen) in the growth media (basal medium was Tadano and Tanno medium) on stimulant production was evident; soybean plants produced larger amounts of germination stimulants when grown under low (1/10) N conditions (6.8 ppm N, upper chromatogram) as compared to the normal N conditions (68 ppm N, lower chromatogram). These results clearly indicated that soybean produced two known strigolactones, orobanchol and alectrol, but not strigol, and their productions seemed to be promoted under low nitrogen availability as in the case of red clover (*11*).

The distribution profiles of germination stimulation activity after ODS-HPLC demonstrated that some of host plants produced novel stimulants as listed in Table I. Among these novel stimulants, there are at least one strigol isomer, two dehydro-strigol isomers, and four tetradehydro-strigol isomers.

Downloaded by COLUMBIA UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch007

False Germination Stimulants; Fungal Metabolites and Jasmonates

Natural germination stimulants such as strigolactones and dihydrosorgoleone are unstable in soil, and no useful and economic suicidal germination stimulants based on these molecules have been obtained, except for promising stimulants developed by Zwanenburg's group at Nijmegen University, the Netherlands (*12*). Germination stimulants structurally unrelated to the natural ones may be important molecular probes to understand the germination mechanism of the parasites, and also to develop useful and economic germination stimulants.

Striga seed germination is also stimulated by other chemicals including natural and synthetic cytokinins, scopoletin, inositol, methionine, sodium hypochlorite, and ethylene. Strigolactones were reported to induce ethylene biosynthesis in *Striga* seeds, and both ethylene biosynthesis and action are required for germination. Therefore, any compounds which stimulate ethylene production may induce *Striga* germination. In fact, 1-aminocyclopropane-1-carboxylate (ACC), the immediate biosynthetic precursor of ethylene, is a good germination inducer for *Striga* seeds. However, ethylene and ACC seem to not be active on *Orobanche* germination (*13*).

Cotylenins and Fusicoccins

Screening of microbial and fungal metabolites for activity as *Striga* and *Orobanche* germination stimulants is probably a more promising strategy. In fact, several fungal metabolites were found to induce *Striga* and *Orobanche* germination. In particular, cotylenins (CNs) and fusicoccins (FCs) produced by *Cladosporium* sp. 501-7W (*14, 15*) and *Fusicoccum amygdalie* Del. (*16, 17*),

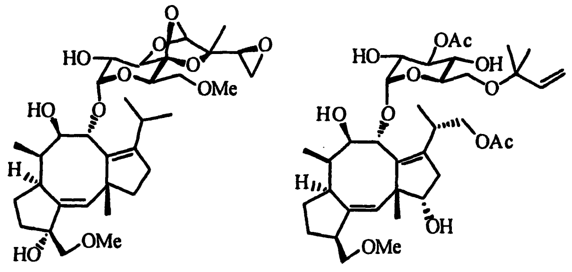
Table I. Germination Stimulants Produced by Various Host Plants

Plant species	Germination Stimulants
Red clover	Orobanchol, alectrol, unknown
Soybean	Orobanchol, alectrol
Cotton	Strigol, strigyl acetate
Carrot	Novel strigolactones
Tomato	Novel strigolactones
Pea	Novel strigolactones
Sorghum	Novel strigolactones, sorgolactone (strigol)
Maize	Novel strigolactones, sorgolactone

Note: Production of strigolactones, except for strigol in sorghum, was confirmed by LC/MS/MS.

respectively, elicited both *Striga* and *Orobanche* seed germination at < 10^{-5} M (*18*). Structures of cotylenin A and fusicoccin A are shown in Figure 4.

Since the structures of CNs and FCs are totally different from those of strigolactones, and, in addition, these fungal metabolites are 1000 to 10000 times less active than strigolactones, they may elicit seed germination by a mechanism different from that by strigolactones. However, in the case of *S. hermonthica* seed germination, both CN- and strigol-induced germinations were reduced by inhibitors of ethylene biosynthesis (aminoethoxyvinylglycine, AVG) and action (sodium thiosulfate, STS), indicating that ethylene is involved in both cases (*18*).



cotylenin A

fusicoccin A

Figure 4. Structures of cotylenin A and fusicoccin A.

Natural and synthetic derivatives of CNs and FCs, fusicoccanes, were then examined to clarify structure-activity relationships in germination stimulation on *O. minor* seeds. In general, structural requirements for parasite germination were similar to those for lettuce seed germination. The substituent on C_9 (R_1) is not necessary for activity and can be H, OH, or an *O*-sugar group (see Figure 5). The substituent on C_{19} (R_2) may be needed for 9-deoxy derivatives. The substituent on C_{12} (R_3) should be a hydroxyl group for derivatives lacking 3-OH (R_4=H) and *vice versa.* Although germination stimulation activities of CNs,

Downloaded by COLUMBIA UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch007

FCs, and their analogues are weak, these compounds, in particular, those lacking 3-OH (FC type compounds), are much more stable than strigolactones in soil, and therefore appropriate structural modifications may afford useful suicidal germination inducers for root parasites (*19*).

In general, *Orobanche* spp. appeared to have stricter germination requirements than *Striga* spp., and the compounds which elicited *Orobanche* germination also induced *Striga* germination. Except for CNs and FCs, only strigolactones have been reported to induce *Orobanche* germination (*1*). In contrast, in addition to CNs, FCs, and strigolactones, *Striga* germination is induced by other chemicals including ethylene, cytokinins, and auxins as mentioned before. Although various plant growth regulators (gibberellins, cytokinins, auxins, abscisic acid, ethylene, and brassinosteroids) have been found not to induce *Orobanche* germination, jasmonate (JA) had not been examined for its effect on the seed germination of these root parasites. Therefore, JA was included in the assay and found to induce the germination of *O. minor (20)*. JA and related compounds were then examined for their effects on the seed germination of *O. minor* and *S. hermonthica*.

Among the compounds examined, esters were more active than the corresponding free acids, and methyl jasmonate (MJA) and 6-*epi*-9,10-dihydrocucurbate were the two most active stimulants (Figure 6). MJA induced more than 50% germination of *O. minor* and *S. hermonthica* seeds at 10^{-4} M. Unfortunately, MJA, JA, and its related compounds are not stable in soil.

Downloaded by COLUMBIA UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch007

Germination Inhibitors

Specific inhibitors of parasite seed germination are useful molecular tools to unveil germination mechanisms. In addition, these inhibitors may be used in the field to reduce germination of parasite seeds if they are stable and safe enough in the environment. Unfortunately, there have been no reports on specific inhibitors of parasite seed germination.

Some fungal metabolites, however, inhibit *Striga* and *Orobanche* germination stronger than those of crop seeds such as lettuce and sorghum. For example, two fungal metabolites, 4,15-diacetoxyscirpenol and 4,15-diacetylnivalenol were isolated as potent germination inhibitors from fungi, probably *Fusarium* spp., contaminated *O. minor* seeds (Figure 7). These compounds inhibited *O. minor* seed germination at < 10^{-5} M without affecting germination and growth of lettuce and maize. Zonno et al. reported *Striga* and *Orobanche* germination inhibition by fungal toxins (*21, 22*). These mycotoxins are highly toxic to mammals and thus these compounds cannot be used as seed killers. However, these toxins may be important as active principles produced by *Fusarium* spp., promising biological control agents of *Striga* and *Orobanche* (*23–25*).

Downloaded by COLUMBIA UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch007

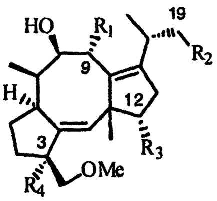


Figure 5. General structures of fusicoccanes.

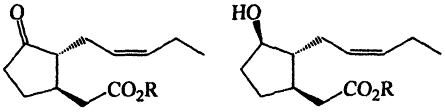
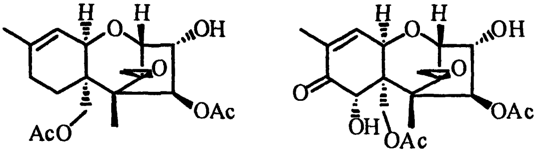


Figure 6. Two most active jasmonate-type germination stimulants.



4,15-diacetoxyscirpenol
4,15-diacetylnivalenol

Figure 7. Fungal metabolites isolated as germination inhibitors for root parasites.

Conclusion

Various plant species have been shown to produce and release germination stimulants for root parasites. Among these germination stimulants, more than 10 compounds are confirmed to be strigolactones by LC/MS/MS analyses, indicating that strigolactones are distributed widely in the plant kingdom. In addition, most host plants examined were found to produce more than one stimulant, and thus these plants may produce individual stimulants at different levels under different growth conditions and/or different growth stages. Once germination stimulants are characterized, chemical analyses like LC/MS/MS may help in screening resistant cultivars for low stimulant production or trap crops for high stimulant production. In addition, some cultivars may be resistant because they produce not only stimulants but also specific inhibitors of germination.

Acknowledgment

Part of the study was supported by Grants-in-Aid for Scientific Research (12460049, 12556015, 15380079) from the Japanese Society for the Promotion of Science (JSPS) and Priority Research Projects, Utsunomiya University.

References

1. Joel, D. M.; Steffens, J. C.; Matthews, D. E. In *Seed Development and Germination*; Kigel, J.; Galili, G., Eds.; Marcel Dekker, Inc.: New York, 1995; pp 567–597.
2. Worsham, A. D. In *Parasitic Weeds in Agriculture*; Musselman, Ed.; CRC Press: Boca Raton, FL, 1987; pp 45–61.
3. Cook, C. E.; Whichard, L. P.; Turner, B.; Wall, M. E.; Egley, G. H. *Science* **1966**, *154*, 1189–1190.

Downloaded by COLUMBIA UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch007

4. Cook, C. E.; Whichard, L. P.; Wall, M. E.; Egley, G. H.; Coggon, P.; Luhan, P. A.; McPhail, A. T. *J. Am. Chem. Soc.* **1972**, *94*, 6198–9199.
5. Siame, B. P.; Weerasuriya, Y.; Wood, K.; Ejeta, G.; Butler, L. G. *J. Agric. Food Chem.* **1993**, *41*, 1486–1491.
6. Hauck, C.; Müller, S.; Schildknecht, H. *J. Plant Physiol.* **1992**, *139*, 474–478.
7. Müller, S.; Hauck, C.; Schildknecht, H. *J. Plant Growth Regul.* **1992**, *11*, 77–84.
8. Yokota, T.; Sakai, H.; Okuno, K.; Yoneyama, K.; Takeuchi, Y. *Phytochemistry* **1998**, *49*, 1967–1973.
9. Sato, D.; Awad, A. A.; Chae, S. H.; Yokota, T.; Sugimoto, Y.; Takeuchi, Y.; Yoneyama, K. *J. Agric. Food Chem.* **2003**, *51*, 1162–1168.
10. Sato, D.; Awad, A. A.; Takeuchi, Y.; Yoneyama, K. *Biosci. Biotechnol. Biochem.* **2005**, *69*, 98-102.
11. Yoneyama, K.; Takeuchi, Y.; Yokota, T, *Physiol. Plantarum* **2001**, *112*, 25–30.
12. Nefkens, G. H. L.; Thuring, J. W. J. F.; Beenakkers, M. F. M.; Zwanenburg, B. *J. Agric. Food Chem.* **1997**, *45*, 2273–2277.
13. Parker, C.; Riches, C. R. *Parasitic Weeds of the World: Biology and Control*; CAB International: Wallingford, Great Britain, 1993.
14. Sassa, T.; Tojyo, T.; Munakata, K. *Nature* **1970**, *227*, 379.
15. Sassa, T. *Agric. Biol. Chem.* **1971**, *35*, 1415–1418.
16. Ballio, A.; Chain, E. B.; De Leo, P.; Erlanger, B. F.; Mauri, M.; Tonolo, A. *Nature* **1964**, *203*, 297.
17. Ballio, A.; Brufani, M.; Casinovi, C. G.; Cerrini, S.; Fedeli, W.; Pellicciari, R.; Santurbano, B.; Vaciago, A. *Experientia* **1968**, *24*, 631–635.
18. Yoneyama, K.; Takeuchi, Y.; Ogasawara, M.; Konnai, M.; Sugimoto, Y.; Sassa, T. *J. Agric. Food Chem.* **1998**, *46*, 1583–1586.
19. Tajima, N.; Kume, H.; Kanematsu, S.; Kato, N.; Sassa, T. *J. Pestic. Sci.* **2002**, *27*, 64–67.
20. Yoneyama, K.; Ogasawara, M.; Takeuchi, Y.; Konnai, M.; Sugimoto, Y.; Seto, H.; Yoshida, S. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 1448–1450.
21. Zonno, M. C.; Vurro, M. *Weed Res.* **1999**, *39*, 15–20.
22. Zonno, M. C.; Vurro, M. *Phytoparasitica* **2002**, *30*, 519–524.
23. Ciotola, M.; Watson, A. K.; Hallett, S. G. *Weed Res.* **1995**, *35*, 303–309.
24. Marley, P. S.; Ahmed, S. M.; Shebayan, A. Y.; Lagoke, S. T. O. *Biocontrol Sci. Tech.* **1999**, *9*, 159–163.
25. Ahmed, N. E.; Sugimoto, Y.; Babiker, A. G. T.; Mohamed, O. E.; Ma, Y.; Inanaga, S.; Nakajima, H. *Weed Sci.* **2001**, *49*, 354–358.

Downloaded by COLUMBIA UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch007

Downloaded by STANFORD UNIV GREEN LIBR on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch008

Chapter 8

Phytotoxins Produced by Invasive Weeds and Their Applications in Agriculture and the Restoration of Natural Areas

Tiffany L. Weir, Laura G. Perry, Jorge M. Vivanco*

Department of Horticulture and Landscape Architecture,
Colorado State University Fort Collins, CO 80523

Plant phytochemicals encompass a great diversity of secondary metabolites that belong to different chemical families produced across the plant kingdom. These phytochemicals have different bioactivities, and in this chapter, we focus on the phytotoxic nature (allelopathy) of some secondary metabolites. We explore different aspects of allelochemistry such as compound identity, modes of action, ecological implications, development of resistance to allelochemicals, and potential uses in agriculture. Furthermore, we highlight areas that are not well understood in allelopathy research and point to some possible solutions.

Use of Plant Derived Compounds for Pest Management

Allelochemistry, or the production and release of toxic chemicals produced by one species that inhibit a receiving species, has the potential to provide a nearly limitless arsenal of natural compounds to be used for pest management. Allelopathy by its broadest definition includes chemicals produced by plants, insects, and microbes. However, merely exploring the allelopathic potential of plant-derived compounds is a daunting task. Higher plants produce a compositionally diverse array of >100,000 low-molecular-mass natural products

In Natural Products for Pest Management; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

known as secondary metabolites (*1*). Many of these secondary metabolites are thought to have evolved partly in response to selective pressures for improved defense mechanisms against a broad assortment of potential threats. Secondary metabolites with allelopathic potential may be present in all plants and can be found in most tissues, including leaves, bark, roots, root exudates, flowers, and fruits (*2*). These chemicals enter the rhizosphere through leaching from aerial plant parts (*3*), volatile emissions (*4*), root exudation (*5,6*), and the breakdown of bark and leaf litter (*3*). Secondary metabolites in the rhizosphere may operate as chemical attractants for beneficial soil microbes and fungi (*7*), deterrents for root herbivores and pathogens (*8*), signals to induce pathogen defense responses (*9*), or phytotoxins to reduce competitive effects of neighboring plants (*5,9*). In addition, plant secondary metabolites may alter soil chemical and physical properties, such as increasing availability of insoluble soil nutrients or creating more favorable conditions for root growth (*10*). Because plant root exudates display such a wide array of potential functions, many may have practical applications in the management of natural and agricultural ecosystems.

Identifying Allelotoxins as Natural Herbicides

There are a number of benefits to identifying and developing plant-produced compounds as tools for pest management, particularly for the control of undesirable plants. Plant-produced phytotoxins, or allelochemicals, have the potential to be more environmentally-benign than traditional synthetic herbicides (*11*). In addition, they are a good source of novel chemicals to replace existing herbicides for which weeds have developed resistance (*12*). Phytotoxins vary widely in their chemical composition and structure, suggesting that they have varying modes of action, although currently the exact mechanisms of many allelochemicals are poorly understood (*13, 14*). Further, allelochemicals cause a variety of physiological effects in susceptible species, with the potential to inhibit seed germination, seedling establishment, and plant survival. Thus, many allelochemicals may operate with mechanisms not present in current commercial herbicides (*12*).

To date, much of the research on plant-produced allelochemicals for weed control has focused on allelochemical production in agricultural crops and cover crops with the aim to develop new cropping systems that control weeds without costly external inputs (*2,15*). However, practical application of these efforts has been limited, primarily by lack of detailed information on the chemicals involved, their modes of action, the genetic, physiological, and environmental controls on their production and activity, and potential mechanisms of resistance in both weeds and crops (*12*). Further, their success may have been limited by the species examined, which have typically yielded only relatively mild

Downloaded by STANFORD UNIV GREEN LIBR on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch008

phytotoxins (*16*). Cultivated plants tend not to exhibit a strong ability to interfere with other plants relative to non-agricultural plants and may not be the most likely species to produce potent phytotoxins.

However, allelochemicals produced by less desirable plants, including invasive species, also have the potential to be used for weed control. Efforts to identify allelochemicals produced by plants capable of intense plant-plant interference may yield a more potent group of natural phytotoxins for use as herbicides. These chemicals could be extracted from plants or generated synthetically and applied exogenously for weed control. More desirable species, such as agricultural crops, could also be genetically engineered to generate a phytotoxic compound, assuming the biosynthetic precursors are already present. Continued research to gain an understanding of the mechanisms of toxicity, genetics of biosynthesis, and controls over production in allelochemicals may allow us to breed or engineer plants with increased allelochemical production (*15*). In addition, understanding mechanisms of resistance to allelochemicals will be critical for identifying chemicals that act selectively against weeds and for breeding or engineering desired species for allelochemical resistance.

Here, we review previous and on-going research on three root-exuded allelochemicals produced by the closely related invasive weeds, *Centaurea maculosa* Lam. (spotted knapweed), *Centaurea diffusa* Lam. (diffuse knapweed) and *Acroptilon* (formerly *Centaurea*) *repens* (L.) DC. (Russian knapweed). We describe the identification of the phytotoxins these species produce and present the existing evidence on modes of action of the phytotoxins, persistence of the toxins in soil, variation among species and genotypes in phytotoxin resistance, and mechanisms of resistance. Further, we discuss the implications of this information with regard to the potential efficacy of the phytotoxins for weed control. Finally, we consider what additional basic information is required to develop effective strategies for using these chemicals as natural herbicides.

Downloaded by STANFORD UNIV GREEN LIBR on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch008

Allelochemical Identification from *Centaurea maculosa*, *Centaurea diffusa*, and *Acroptilon repens*

Centaurea maculosa, *C. diffusa*, and *A. repens,* members of the Asteraceae family, were introduced to North America in the early 20th century presumably as contaminants in alfalfa and clover seed, and have since spread throughout the western United States and Canada. In 1988, *C. maculosa* occupied over 2.8 million hectares in North America (*17*) and has now become the largest rangeland weed problem in western Montana (*18*). *C. diffusa* occupies over 1.9 million hectares in North America (*17*). *Acroptilon repens* occupies over 43,000 hectares in Colorado and Montana, and heavy infestations were also reported in eastern Washington and Oregon and in southern Idaho in 1985 (*19*). These plants often establish monocultures in their new habitats (*20*), resulting in lower native species diversity (*21*), increased soil erosion (*22*), and reduced livestock and wildlife forage quality (*23,24*).

Ecological Evidence of Allelopathy in Knapweeds

Although the knapweeds are highly successful in North America, they tend to be relatively minor components of plant communities in their native range. The predominant theory explaining the success of invasive species in new habitats is that they are able to compete more successfully for limited resources because they have evaded their natural enemies (*25*). However, production of phytotoxic allelochemicals, novel to North American plants, has been proposed as an alternative explanation for the success of these invasive knapweeds (*26*). These "novel weapons" (*27*) would be expected to have relatively little effect in Europe, where neighboring plants would have co-evolved resistance to them, but may have large effects in North America, accounting for the rapid displacement of native plant communities by knapweeds. Callaway and Aschehoug (26) first reported evidence that allelopathy contributes to the invasive success of *C. diffusa* using experiments in which activated carbon was added to soil to adsorb organic compounds. *Centaurea diffusa* showed stronger negative effects on grass species from North America than on European congeners from communities where *C. diffusa* is native, but the effect on North American grasses was alleviated by addition of activated carbon. This suggested that the negative effects of *C. diffusa* on North American grasses were primarily due to organic compounds produced and secreted in *C. diffusa* root exudates. Similar experiments with *C. maculosa* indicated that root exudates also facilitate *C. maculosa* dominance over native North American grasses (*21*). When competing with some North American species, the *Centaurea* spp. grew less in the presence of activated carbon, suggesting that removal of allelopathy shifts the balance of competition in favor of the North American species (*21, 26*).

Centaurea maculosa, *Centaurea diffusa*, and *Acroptilon repens* Produce Different Allelochemicals

While evidence suggested that *Centaurea* invasions had an allelopathic mechanism (*21, 26*) no candidate phytotoxic metabolite had been successfully isolated or characterized from these plants. To address this issue Bais et al. (*28*) developed a system where root exudates of *in vitro*-grown *C. maculosa* were collected in sterile MS media. Collected root exudates were assayed to determine phenotypic effects and effects on germination efficiency of several

Downloaded by STANFORD UNIV GREEN LIBR on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch008

weeds, including *C. diffusa*, *Linaria dalmatica* (L.) P. Mill., *Verbascum thapsus* L., *Bromus tectorum* L, *Kochia scoparia* (L.) Schrad, the model plant *Arabidopsis thaliana* (L.) Heynh., and crop plants such as wheat (*Triticum aestivum* L.) and tomato (*Lycopersicon esculentum* L.). All plants exhibited decreased root and shoot growth and showed mortality within 14 days of exposure to *C. maculosa* root exudates. The individual components of these root exudates were separated using high-pressure liquid chromatography (HPLC) and the phytotoxic fraction was identified as a racemic mixture of (±)-catechin by mass spectrometry (MS) and nuclear magnetic resonance (^{1}H and ^{13}C NMR) (*5*). Further examination revealed that the (-)-catechin component was primarily responsible for phytotoxicity while (+)-catechin had only a mild phytotoxic effect but displayed broad-spectrum antimicrobial activity (*5,43*). A similar procedure was followed to identify *C. diffusa's* root secreted phytotoxin as *8*-hydroxyquinoline (*29*) and to determine that *A. repens* roots exude the phytotoxin *7,8*-benzoflavone (*30*).

Downloaded by STANFORD UNIV GREEN LIBR on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch008

Structure and Activity of the Knapweed Allelochemicals

Surprisingly, the three phytotoxins differ greatly in chemical structure (Figure 1), despite the close relationship of the three knapweeds (*30*). (+)-Catechin, a widespread plant flavanoid, is a well-characterized antioxidant free radical scavenger, reported as a major component of green tea (*31*). Its reported properties include activity as an antitumour agent (*32*) and insect repellent (*33*). In nature, the (+) isomer and racemic form of catechin occur more frequently than the (-)-isomer (*34, 35*). 7,8-Benzoflavone and 8-hydroxyquinoline have not been previously reported as natural products (*29, 30*), although 7,8-benzoflavone reportedly activates cytochrome P450 (*36*), inhibits DNA damage attributed to benzoprenes (*37, 38*), and binds to adenosine receptors (*39*), while 8-hydroxyquinoline is known to inhibit the enzymatic activity of plant alternative oxidase (AOX) protein (*40*). 7,8-benzoflavone is the least potent of the three compounds, requiring higher concentrations to have the same phytotoxic effect on *Arabidopsis thaliana* as catechin or 8-hydroxyquinoline (*29*).

Precursors in the Flavonoid Pathway Do Not Exhibit Phytotoxicity

The varied structures and published activities of the three identified phytotoxins suggest that they have different cellular targets. However, the differing phytotoxicity between the (+)- and (-)- isomers of catechin provide a unique opportunity to explore the relationship between structure and function of these molecules. Under the experimental conditions described by Bais et al (*41*), the (+)-catechin isomer showed no phytotoxic activity, while several compounds and intermediates of the flavonoid pathway, such as naringenin, kaempferol, quercetin, (-)-epicatechin, (+)-epicatechin, and (±)-dihydroquercetin showed little effect on root and shoot growth and general phytotoxic activity relative to (-)-catechin (*41*). Only (+)-epicatechin, another relatively rare naturally occurring flavonoid (*42*) was phytotoxic to *C. maculosa* (*41*). The (+) isomer of epicatechin differs from (-)-catechin only in that it is found in a 2,3-*cis* relationship as opposed to a 2,3-*trans* configuration, indicating that it is the spatial arrangement of (-)-catechin that is specifically recognized and excluded from internalization by *C. maculosa*. Indeed, *C. maculosa* suffers no ill effects from exogenous exposure to relatively high concentrations of (-)-catechin, but microinjection of the chemical into the root tip cells is deadly (*9*). This also suggests that it is the 2*S* configuration, present in both molecules, that is important for phytotoxicity.

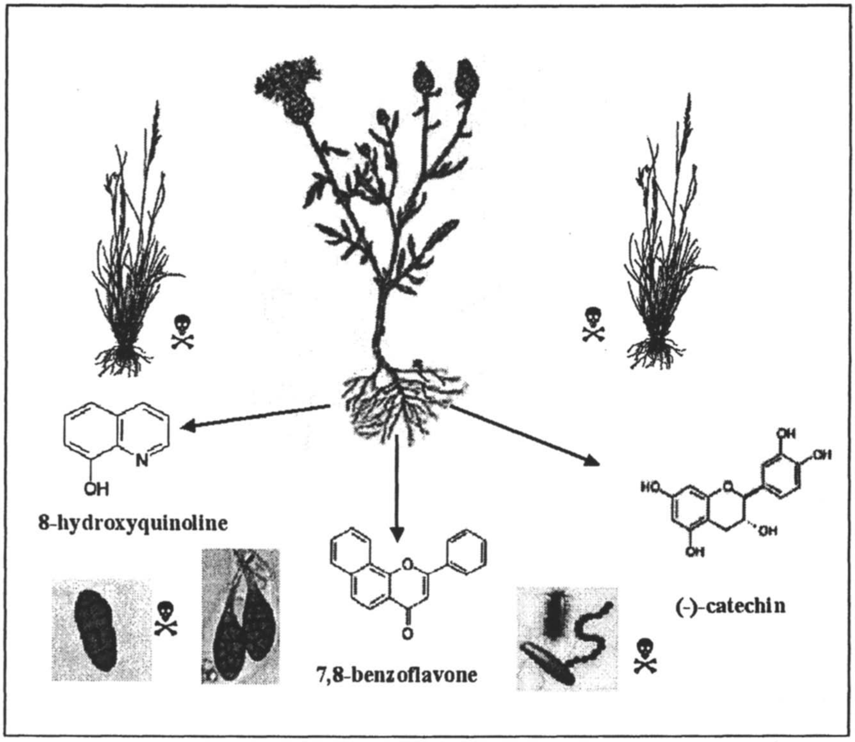


Figure 1. Three invasive knapweeds in western North American secrete three different allelochemicals. All of these allelochemicals were determined to be phytotoxic, while the (+)-isomer of racemic catechin and 8-hydroxyquinoline were found to be antimicrobial as well.

Downloaded by STANFORD UNIV GREEN LIBR on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch008

Structural Modifications of (-)-Catechin Provide Insights into Activity

Further studies into the structure/function relationship of (+)- and (-)-catechin isomers were conducted by synthetically modifying the hydroxyl groups that provide the antioxidant properties of catechin. (+)- and (±)-Pentaacetyl, tetramethoxy, and 6*a*,12a-*trans*-2,3,8,10-Tetra-hydroxy-5,5-dimethyl-5,6*a*,7,12*a*-tetrahydro[1]benzopyrano[3,2-*c*], a cyclized derivative, were synthesized and tested for phytotoxicity against a number of plant species including *C. maculosa.* (±)-Tetramethoxycatechin and the (±)-cyclized derivative showed phytotoxicity comparable to that of (-)-catechin and inhibited root differentiation in *C. maculosa* (*43*). These compounds are much less polar than (-)-catechin, which may facilitate their uptake across hydrophobic cell walls and membranes, allowing them to evade *C. maculosa's* mechanism for excluding cellular uptake of catechin. The fact that the (±)- and not the (+)-derivatives were phytotoxic strengthens the hypothesis that the 2*S* configuration is important for phytotoxicity, but indicates that slight modification of the C-2/C-2' region by cyclization is not detrimental. These results also minimize the importance of the antioxidant free hydroxyl groups in (-)-catechin phytotoxicity.

Downloaded by STANFORD UNIV GREEN LIBR on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch008

Investigation of the Mode of Action of (-)-Catechin

Microinjection of (-)-catechin into *C. maculosa* (*9*) and structural modifications of (-)-catechin leading to autotoxicity (*43*) suggest that its phytotoxicity relies at least partly on internalization of the chemical by susceptible species. However, the intracellular target and immediate physiological response of affected plants was still unclear. Bais et al (*9*) examined these questions by monitoring the generation of signaling molecules in susceptible plant species. They monitored fluxes in reactive oxygen species (ROS) and cytoplasmic calcium ($[Ca^{2+}]_{cyt}$) in response to exogenous application of (-)-catechin in *C. maculosa*, *C. diffusa*, and *A. thaliana*. *C. maculosa* did not initiate any signaling cascade in response to the (-)-catechin treatment, but both susceptible species showed transient increases in both ROS and $[Ca^{2+}]_{cyt}$. A wave of ROS moved from the root meristematic tissue and back through the root tip to the central elongation zone within 10 seconds of (-)-catechin treatment. This was followed by a rapid but transient increase in $[Ca^{2+}]_{cyt}$ localized at the root tip, and resulted in a dramatic drop in cytoplasmic pH. By 5-10 minutes loss of cell viability, as determined by leakage of fluorescence from cells stained with fluorescein diacetate (FDA), began to occur and followed the same spatial kinetics of the ROS induction. However, a concurrent treatment of (-)-catechin and ascorbic acid, an antioxidant, did not result in the activation of the signaling cascade in *C. diffusa* and *A. thaliana*, while treatment of these plants with hydrogen peroxide did trigger the cascade (*9*). This implies that the phytotoxicity of (-)-catechin is partially due to its ability to generate free radicals in the plant and that the ROS/ $[Ca^{2+}]_{cyt}$ signal is an essential prerequisite for (-)-catechin-mediated cell death.

Arabidopsis Provides a Genetically Tractable Model for Studying the Cellular Effects of (-)-Catechin

To further examine the physiological effects of (-)-catechin on susceptible species, Bais et al (*9*) utilized the genomic tools available for studying *A. thaliana* to analyze global gene expression in response to (-)-catechin treatment. Ten minutes after treatment with (-)-catechin, 10 genes that are implicated in oxidative stress-activated signal transduction events were upregulated, including peroxidase ATP21a and calmodulin, a calcium-binding gene. A gene associated with a steroid sulfotransferase-like protein, α-cystathionase, a chlorophyll binding protein, a ribosomal protein L9, and four genes lacking homology with known genes and of undetermined function were also upregulated. After an hour, nearly 1,000 genes were upregulated, many of which are also activated in response to oxidative stress, including glutathione transferase (GST), monooxygenase, lipid transfer protein, heat shock protein (HSP), and blue copper binding protein. Genes encoding enzymes of the phenylpropanoid (*LDOX*) and terpenoid phytoalexin pathway (*VS*) were also among those induced. While metabolites produced by these enzymes are thought to function primarily for defense reactions against pathogens and UV, many of them also act as antioxidants (*44*). Rather than having a specific intracellular target, the array of upregulated oxidative stress related genes supports the idea that generation of oxidative free radicals in the plant is largely responsible for the phytotoxicity of (-)-catechin. Interestingly, ROS have been implicated in both plant defense and plant stress (*45*). They reportedly play a role in signal transduction events that trigger defense mechanisms, such as the hypersensitive response. However, in some compatible host/pathogen interactions, ROS accumulate in plant cells causing damage such as depolarization of cell membranes, lipid peroxidation, DNA damage, and eventually leading to cell death (*13*).

Soil Allelochemical Concentrations

An important consideration for the development of allelochemicals as natural products for weed control is their concentration, activity, and persistence in soil. Several arguments against the importance of allelopathy in plant interactions have questioned whether sufficient quantities of phytotoxic chemicals are produced and maintained in the soil to affect recipient plants, and whether the microbiological and physical properties of the soil affect the

Downloaded by STANFORD UNIV GREEN LIBR on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch008

persistence or activity of these compounds (*46*, *47*, *48*). Published measurements of (±)-catechin concentrations in soil from North American *C. maculosa* populations vary from 0.3 to over 1.2 mg g^{-1} dry soil (*8*, *9*). More recent measurements have indicated soil (±)-catechin concentrations as high as 7 mg g^{-1} (L. Perry, unpublished data). In experiments in which (-)-catechin was added to soil, 0.2 mg g^{-1} was sufficient to inhibit North American grassland species germination and growth (*9*). Likewise, published measurements of soil 8-hydroxyquinoline concentrations in North American *C. diffusa* populations vary from 200 to over 260 μg g^{-1}, while only 40 μg g^{-1} was required to inhibit plant growth in greenhouse experiments (*30*). Although (-)-catechin and 8-hydroxyquinoline may have different activity when produced in nature than when added in experimental settings, these results suggest that soil allelochemical concentrations maintained in *C. diffusa* and *C. maculosa* populations are sufficiently large for allelopathy to play a major role in plant interactions. Whether these high soil concentrations are due to slow degradation rates or high rates of production is not known. Soil 7,8-benzoflavone concentrations in *A. repens* populations have not yet been examined. Developing knapweed allelochemicals for weed control will require information on the concentrations to apply and on the effectiveness of foliar applications compared to soil amendments. Since root-exuded allelochemicals typically influence plant roots the latter is more likely to be the best method of application.

Downloaded by STANFORD UNIV GREEN LIBR on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch008

Added Benefits of Allelochemicals: Effects on Soil Pathogens

Another advantage of exploring the potential of plant products for pest control is that a number of the compounds produced may have multiple uses. Root exudation represents a significant carbon cost to the plant; nearly 5-21% of photosynthetically fixed carbon is deposited into the soil as root exudates (*49*). Thus, it stands to reason that at such a high cost to the plant, a number of these deposited exudates would serve multiple functions in the rhizosphere. Accordingly, both (±)-catechin and 8-hydroxyquinoline exhibit antimicrobial activity. 8-Hydroxyquinoline treatments lead to decreased hyphal growth of several pathogenic fungi and reduced cell density counts of several types of soil-borne pathogenic bacteria (*30*). (-)-Catechin appears to have no effect on bacteria, but the concurrently secreted (+)-catechin isomer has been shown to reduce growth of gram-negative plant pathogenic bacteria (*8*, *43*). Interestingly, all of the catechin derivatives tested by Veluri et al. (*43*) also had antimicrobial properties, although they were slightly less active relative to (+)-catechin. The antimicrobial activity of 7,8-benzoflavone has not yet been tested. The antimicrobial activity of both (+)-catechin and 8-hydroxyquinoline suggests that *C. maculosa* and *C. diffusa* invasions may be mediated by chemically induced changes in soil microbial communities as well as by allelopathic interactions.

Experiments examining effects of soil sterilization on competition between *Centaurea* spp. and grassland species in North American and European soils indicate that soil microbial communities play a role in both *C. maculosa* and *C. diffusa* invasions (*30, 50*). Finally, the antimicrobial activity of (+)-catechin and 8-hydroxyquinoline suggests a potential additional utility of these compounds for controlling soil-borne plant pathogens in managed ecosystems.

Selectivity of Allelochemicals for Weed Control

To date, practical applications of plant-produced phytotoxins for weed control have been limited in part by a lack of potency of identified allelochemicals (*16*). The substantially greater sensitivity of North American species than European species to (-)-catechin and 8-hydroxyquinoline highlights the importance of novelty to the potency of plant-produced allelochemicals (*9, 26, 30*). The tendency for plants to evolve resistance to the allelochemicals produced by their competitors suggests that the search for effective natural allelochemicals for weed control may be most fruitful when focused on allelochemicals produced by plants to which the target weeds have never been exposed.

Mechanisms of Resistance

Understanding potential mechanisms of tolerance and resistance to natural herbicides will be critical to evaluating where we can employ these compounds effectively to favor desired species over target weeds. To be effective, allelochemical herbicides must be applied in systems where the desired species are naturally insensitive, or where the desired species have been bred or engineered for resistance. In particular, in systems where the desired species are bred or engineered to produce allelochemicals, breeding or engineering for resistance will also often be necessary. In addition, understanding mechanisms of allelochemical resistance will be important for understanding how allelochemical resistance might develop in target weeds, and may provide insights into allelochemical modes of action. Research on detoxification of some allelopathic compounds indicates that resistant plants may detoxify allelochemicals via carbohydrate conjugation, oxidation, or sequestration (*13, 14*). For example, plants insensitive to benzoxazolin-1 (3*H*)-one (BOA) detoxify this allelopathic compound through *N*-glucosylation, addition of a pentose sugar moiety, or through hydroxylation followed by glucosylation (*51, 52*), resulting in structurally similar but less toxic compounds that are released into the rhizosphere (*53*). One recent report suggests that another detoxification mechanism is the secretion of an enzyme, laccase, which catalyzes the oxidation

Downloaded by STANFORD UNIV GREEN LIBR on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch008

of phenolic compounds. A transgenic *A. thaliana* that overexpressed a *Gossypium arboreum* LAC1 gene exhibited enhanced resistance to several phenolic allelochemicals (*54*).

Experiments testing the effects of (-)-catechin, 8-hydroxyquinoline, and 7,8-benzoflavone on plant growth have revealed substantial variation in allelochemical sensitivity among North American species. For example, (-)-catechin treatment substantially reduced germination of two North American grassland species, *Festuca idahoensis* and *Koeleria macrantha*, but reduced *K. macrantha* growth to a greater extent than *F. idahoensis* growth (*9*). In a second experiment, the (-)-catechin concentrations required to reduce root growth and survival were much lower for *F. idahoensis*, *K. macrantha* and *Nassella tenuissima* (Trin.) Barkworth (10 to 50 $\mu g\ ml^{-1}$) than for *Gaillardia aristata* and the hybrid *G.* x. *grandiflora* Van Houtte (*55*). *Gaillardia aristata* was also resistant to 7,8-benzoflavone compared to a variety of other species (*29*). Variation among species in response to knapweed allelochemicals suggests that it may be possible to employ these compounds selectively as herbicides in systems in which the desired species are naturally insensitive to them. In addition, identifying native North American species that are insensitive to knapweed allelochemicals could be helpful in the restoration of invaded grasslands and creation of plant communities that are less vulnerable to new invasions. Experiments are currently underway to identify additional native North American grassland species that are particularly insensitive to catechin (L. Perry, unpublished data) and to determine mechanisms of resistance to catechin in North American plants (T. Weir, unpublished data).

Some native North American species may be evolving resistance to knapweed allelochemicals. Mealor and colleagues (*56*) found that offspring from North American populations invaded more recently by *A. repens* are more susceptible to *A. repens* interference than offspring from long-invaded North American populations. They grew the plants for two generations in a common garden to remove maternal effects, thus demonstrating that the observed differences were evolved. Whether their results reflect increased resistance to 7,8-benzoflavone has not yet been examined. Rays results here ()Evolution of resistance to knapweed allelochemicals in North American populations would demonstrate the potential for rapid evolution of allelochemical resistance and would suggest that knapweed competitive dominance in North American grasslands may decrease with time. Further, such evolution would indicate that breeding may have the potential to be an effective method for generating allelochemical-resistant crops. However, it would also indicate that weeds are likely to quickly develop resistance to allelochemicals employed as herbicides. Studies to examine the mechanisms of resistance may yield insights into appropriate strategies for developing or avoiding allelochemical resistance.

Downloaded by STANFORD UNIV GREEN LIBR on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch008

Conclusions

The available evidence on *C. maculosa*, *C. diffusa*, and *A. repens* allelochemicals suggests that they are potent, selective phytotoxins with the potential to be maintained at high concentrations in soil. In addition, research on the modes of action and mechanisms of resistance to (-)-catechin, one of the allelochemicals, suggests avenues for enhancing the selectivity of (-)-catechin to control weeds but not other plants. Research is needed to address many practical issues before knapweed allelochemicals can be employed as natural herbicides, including developing methods for application in agricultural or natural systems, determining the duration of activity once the chemicals are applied to the soil, assessing effects of environmental conditions on allelochemical potency and longevity, and identifying particularly sensitive weed species or resistant crops or native species. Developing effective strategies for using knapweed allelochemicals for weed control will first require a more complete understanding of how these allelochemicals act to inhibit germination and growth of susceptible species and of how resistant species avoid effects of the allelochemicals.

Downloaded by STANFORD UNIV GREEN LIBR on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch008

References

1. Dixon, R. A. *Nature.* **2001,** *411*, 843-847.
2. Weston, L. A. *Agron. J.* **1996,** *88*, 860-866.
3. Inderjit; Keating, K. I. *Adv. Agron.* **1999,** *67*, 141-231.
4. Steeghs, M.; Bais, H. P.; de Gouw, J.; Goldan, P.; Kuster, W.; Northway, M.; Fall, R.; Vivanco, J. M. *Plant Physiol.* **2004,** *135*, 47-58.
5. Bais, H. P.; Walker, T. S.; Stermitz, F. R.; Hufbauer, R. A.; Vivanco, J. M. *Plant Phys..* **2002,** *128*, 1173-1179.
6. Walker, T. S.; Bais, H. P.; Grotewold, E.; Vivanco, J. M. *Plant Physiol.* **2003,** *132*, 44-51.
7. Fons, F.; Amellal, N.; Leyval, C.; Saint-Martin, N.; Henry, M. *Can. J. Microbiol.* **2003,** *49*, 367-373.
8. Walker, T. S.; Bais, H. P.; Deziel, E.; Schweizer, H. P.; Rahme, L. G.; Fall, R.; Vivanco, J. M. *Plant Physiol.* **2004,** *134*, 320-331.
9. Bais, H. P.; Vepachedu, R.; Gilroy, S.; Callaway, R. M.; Vivanco, J. M. *Science.* **2003,** *301*, 1377-1380.
10. Wardle, D. A.; Nilsson, M.; Gallet, C.; Zackrisson, O. *Biological Reviews of the Cambridge Philosophical Society.* **1990,** *73*, 305-319.
11. Macias, F. A. In *Allelopathy: Organisms, Processes, and Applications*; Inderjit; Dakshini, K. M. M.; Einhellig, F. A. Eds.; American Chemical Society, Washington, DC, 1995.
12. Vyvyan, J. R. *Tetrahedron.* **2002,** *58*, 1631-1646.

13. Weir T. L.; Park, S.-W.; Vivanco, J. M. *Current Opinion in Plant Biology.* **2004,** 7, 472-479.
14. Inderjit; Duke, S. O. *Planta.* **2003,** *217*, 529-539.
15. Foley, M. E. In *Expanding the Context of Weed Management*; Buhler, D. D. Ed.; Food Products Press, The Haworth Press, Inc: New York, NY, 1995.
16. Duke, S. O.; H. K., Abbas. In *Allelopathy: Organisms, Processes, and Applications.* Inderjit; Dakshini, K .M. M.; Einhellig, F. A., Eds.; American Chemical Society: Washington, D.C., 1995.
17. Muller-Schärer, H.; Schroeder, D. *Pesticide Sci.* **1993,** *37*, 343-353.
18. *Weeds of the West.* Whitson, T. D.; Burrill, L. C.; Dewey, S. A.; Cudney, D. W.; Nelson, B. E. et al. Eds.; Pioneer of Jackson Hole: Jackson, WY, 1999.
19. Maddox, D. M.; Mayfield, A.; Poritz, N. H. *Weed Sci.* **1985,** *33*, 315-327.
20. Callaway, R. M. *Trends in Ecology and Evolution.* **2002,** *17*, 104-105.
21. Ridenour, W. M.; Callaway, R. M. *Oecologia.* **2001,** *126*, 444-450.
22. Lacey, J. R.; Marlow, C. B.; & Lane, J. R. *Weed Technol.* **1989,** *3*, 627-631.
23. Thompson, M. J. *Northwest Scientist.* **1996,** *70*, 10-19.
24. Jacobs, J. S.; Sheley, R. L. *Great Basin Naturalist.* **1999,** *59*, 175-181.
25. Keane, R. M.; Crawley, M. J. *Trends in Ecology and Evolution.* **2002,** *17*, 164-170.
26. Callaway, R. M.; Aschehoug, E. T. *Science.* **2000,** *290*, 521-523.
27. Hierro, J. L.; Callaway, R. M. *Plant and Soil.* **2003,** 256, 29-39.
28. Bais H. P; Loyola-Vargas, V. M.; Flores H. E.; Vivanco, J. M. *IN VITRO Growth & Development-Plant.* **2001,** *37*, 730-741.
29. Stermitz, F. R.; Bais, H. P.; Foderaro, T. A.; Vivanco, J. M. *Phytochemistry.* **2003,** *64*, 493-497.
30. Vivanco, J. M.; Bais, H. P.; Stermitz, F. R.; Thelen, G. C.; Callaway, R. M. *Ecol. Lett.* **2004,** *7*, 285-292.
31. Kim, S. J.; Han, D.; Ahn, B. H.; Rhee, J. S. *Bioscience, Biochemistry, and Biotechnology.* **1997,** *61*, 225-229.
32. Du, Q.; Cheng, H.; Ito, H. *J. Chromatogr.* **2001,** *921*, 331-334.
33. Kiderlen, A. F.; Kayser, O.; Ferreira, D.; Kolodziej, H. *Z Naturforsch.* **2001,** *56*, 444-54.
34. Nahrstedt, A.; Proksch, P.; Conn, E. E. *Phytochemistry.* **1987,** *26*, 1546-1546.
35. Karimdzhanov, A. K.; Kusnetsova, N. N.; Dzhataev, S. A. *Chemistry of Natural Compounds.* **1997,** *33*, 250-253.
36. Lee, C. A.; Manyike, P. T.; Thummel, K. E.; Nelson, S. D.; Slattery, J. T. *Drug. Metab. Disp.* **1997,** *25*, 1150-1156.
37. Baird, W. M.; Diamond, L. *Chem.-Biol. Interact.* **1976,** *13*, 67-75.
38. Wu, C.-H.; Hsieh, C.-L., Song, T.-Y.; Yen, G.-C. *J. Agric. Food Chem.* **2001,** *49*, 2579-2586.
39. Ji, X.-D.; Melman, N.; Jacobsen, K. A. *J. Med. Chem.* **1996,** *39*, 781-788.

Downloaded by STANFORD UNIV GREEN LIBR on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch008

40. Affourtit, C.; Moore, A. L. *Biochim. Biophys. Acta - Bioenergetics.* **2004,** *1608,* 181-189.
41. Bais H. P.; Walker T. S.; Kennan A. J.; Stermitz F. R.; Vivanco, J. M. *J. Agric. Food Chem.* **2003b**, *51*, 897-901. Erratum published in *J. Agric. Food Chem.* **2004,** *52,* 7746.
42. Della Monache, F.; Ferrari, F.; Poce-Tucci, A.; Marini-Bettolo, G. B. *Phytochemistry* **1972,** *11,* 2333-2335.
43. Veluri, R.; Weir, T. L.; Bais, H. P.; Stermitz, F. R.; Vivanco, J. M. *J. Agric. Food Chem.* **2004,** *52,* 1077-1082. Erratum published in *J. Agric. Food Chem.* **2004,** *52,* 7746.
44. Sticher, L.; Mauch-Mani, B.; Metraux, J. P. *Ann. Rev. Phytopath.* **1997,** *35,* 235-270.
45. Huckelhoven, R.; Kogel, K. H. *Planta.* **2003,** *216,* 891-902.
46. Harper, J. L. *Q. Rev. Biol.* **1975,** *50,* 493.
47. Makino, T.; Takahashi, Y.; Sakurai, Y.; Nanzyo, M. *Soil Sci. Plant Nutr.* **1996,** *42,* 867-879.
48. Weidenhamer, J. D.; Romeo, J. T. *J. Chem. Ecol.* **2004,** *30,* 1067-1082.
49. Marschner, H. *Mineral Nutrition of Higher Plants;* 2nd edition. Academic Press, London, UK, 1995.
50. Callaway, R. M.; Thelen, G. C.; Rodriguez, A.; Holben, W. E. *Nature.* **2004,** *427,* 731-733.
51. Weiland, I.; Kluge, M.; Schneider, B.; Schmidt, B.; Sicker, D.; Schulz, M. *Phytochemistry* **1998,** *49,* 719-722.
52. Weiland, I.; Freibe, A.; Kluge, M.; Sicker, D.; Schulz, M. In *Recent Advances in Allelopathy. A Science for the Future,* Vol. 1. Macias et al., Eds.; S.A.I. Universidad de Cadiz, Madrid, Spain. 1999 pp. 47-56.
53. Sicker, D.; Schneider, B.; Hennig, L.; Knop, M.; Schulz, M. *Phytochemistry.* **2001,** *58,* 819-825.
54. Wang, G. D.; Li, Q. J.; Luo, B.; Chen, X. Y. *Nat. Biotechnol.* **2004,** *22,* 893-897.
55. Weir, T. L.; Bais, H. P.; Vivanco, J. M. *J. Chem. Ecol.* **2003,** *29,* 2397-2412.
56. Mealor, B. A.; Hild, A. L.; Shaw, N. L. *Western North American Naturalist.* **2004,** 64 (4) *in press.*

Downloaded by STANFORD UNIV GREEN LIBR on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch008

Chapter 9

Isolation Strategies for Finding Bioactive Compounds: Specific Activity vs. Total Activity

Syuntaro Hiradate

Department of Biological Safety Science, National Institute for Agro-Environmental Sciences (NIAES), Tsukuba, Ibaraki 305-8604, Japan

Downloaded by CORNELL UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch009

There are two major strategies for isolation of bioactive natural products. One is to find compounds with high *specific activity* (biological activity per unit weight of the compound). This leads to the discovery of the most active compounds present in an organism with low EC_{50} (effective concentration of the compound to induce half-maximum action). In the other strategy, compounds with high *total activity* (biological activity per unit weight of the organism containing the bioactive compound), causing a particular phenomenon, are isolated. In this strategy, EC_{50} of the compound is not necessarily low. The total activity is determined by the specific activity and concentration (or content) of the compound in the organism, and this could explain the role and influence of the compound in the phenomenon. Differences between the two strategies and the importance of the choice for the right one to apply are emphasized, and applicatory examples complementary to each strategy are presented. The author proposes to use the terminology and concept of "total activity" and "specific activity" to avoid confusion in scientific discussions.

It is well-known that organisms contain bioactive compounds to control the growth of the organism itself (e.g., phytohormones) or that of the other organisms (e.g., allelochemicals). In general, these compounds are environmentally less-toxic because the natural ecosystem has an enzymatic system to degrade and/or recycle them. A great deal of effort has been made to utilize these chemicals for pest and weed management.

To utilize bioactive natural products, the effective components have been isolated from crude extracts of the target organisms. The isolated compounds have been characterized in terms of their chemical structures, toxicities to organisms, and environmental safety, etc. It might be possible that these bioactive compounds are chemically modified, industrially synthesized, and manufactured as active ingredients (chemical use). In recent decades, however, the ways of utilizing natural compounds have become diversified. For example, microbes that secrete bioactive compounds are directly applied as bio-agrochemicals (biological use). It is also possible that crude extracts of active organisms are applied as agrochemicals without thorough purification. The isolation strategy for finding bioactive compounds therefore depends on the utilization strategy (chemical use or biological use). In some cases, however, the isolation strategy and the utilization strategy are not consistent with each other, and this leads to confusion.

In the following sections, concepts of the isolation strategies for finding bioactive compounds are summarized using two terminologies, "*total activity*" and "*specific activity*", and the importance of the choice for the right isolation strategy to apply is emphasized. The specific activity will be more important for searching new pesticides than the total activity, and chemical ecologists will be more interested in the total activity. Technical application and some research examples complementary to each strategy are also presented. The author proposes to use these terminologies and concepts in scientific discussions for clarity.

Downloaded by CORNELL UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch009

Concepts of "Specific Activity" and "Total Activity"

Isolation of bioactive natural products can be separated up into two strategies: (1) find compounds with high *specific activity* for chemical use (specific activity strategy), and (2) find compounds with high *total activity* for biological use and for isolation of compounds causing a particular phenomenon (total activity strategy).

Specific Activity

In general, biological activity of a compound is expressed by EC_{50}, which is the effective concentration of a compound to induce half-maximum action.

Because this activity is expressed based on the specific concentration of the compound, it should be termed as *specific activity*.

A compound with a high specific activity (low EC_{50}) could be used as a pesticide per se, or as a prototype for the development of synthetic analogs (chemical use). This kind of research is common and has been successfully applied. For example, some natural phytohormones, such as gibberellins and brassinosteroids, are industrially synthesized and used as commercial plant growth regulators. Phosphinothricylalanylalanine (bialaphos), a commercial natural herbicide, is a fermentation product isolated from cultures of *Streptomyces hygroscopicus*. These compounds have high specific activities.

Total Activity

Assume a case that a crude extract of an organism showed a significant herbicidal activity, and it is to be used without thorough purification. To isolate the herbicidal compounds, the specific activity alone would not be a good indicator for the purification, but both the specific activity and concentration (or content) of the compound in the extract should be considered. Assume a case in which a crude extract contains two herbicidal compounds, A and B (Figure 1).

Crude Extract

	Specific Activity (EC_{50}, *μM*)	Concentration (*μM*)	Total Activity (No Unit)
Ⓐ	1	1	1
$\boxed{B}$	10	100	10

Specific Activity : **A** > **B** (10-fold higher in **A**)
Concentration : **B** > **A** (100-fold higher in **B**)
Total Activity : **B** > **A** (10-fold higher in **B**)

Total Activity = Concentration (*μM*) / Specific Activity (EC_{50} in *μM*)

Figure 1. An example of a crude extract containing two bioactive compounds, ***A*** *and* ***B****. Which compound do you need to isolate?*

Downloaded by CORNELL UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch009

Compound A has 10-fold higher specific activity than compound B, but the concentration of compound A is 1/100 of compound B. Which compound needs to be isolated? Of course, it depends on the purpose of the experiment. In this case, wherein the causal compound is the target, compound B should be isolated as the most influential compound, because compound B is estimated to cause 10-fold stronger effect than compound A. But if the specific activity will be taken as an indicator in the purification procedures, compound A, which is the wrong target, will be the one being isolated instead. Therefore, it is very important to select the right isolation indicator.

Here, the author proposes *total activity* as defined follows:

$$\text{total activity} = (\text{concentration or content}) / \text{specific activity } (EC_{50}) \quad (1)$$

Because the concentration (or content) of the compound and the specific activity have same dimension, the total activity has no unit. The value of the total activity obtained by equation 1 expresses the dilution ratio of the organism (or original material) to obtain the EC_{50}. An organism (or its extract) with high total activity has a high potential to be influential in nature. A compound with a high total activity does not necessarily have a high specific activity (low EC_{50}). The total activity is an indicator of the influence of a compound in a mixed solution or in an organism, and is applied for isolating the causal compound of a particular phenomenon.

Methods to Follow the Specific and Total Activities

In this section, isolation procedures based on the specific and total activities are described.

Isolation Procedure to Follow the Specific Activity as an Indicator

Figure 2 shows a typical isolation procedure to follow the specific activity (inhibitory activity), assuming the following case. An original material (or organism) is extracted with a solvent, and the weight of the extracted ingredients (A kg) is measured after the removal of the solvent. Dissolving A *kg* of the extracted material into 1 L of solvent results a concentration of A kg extract / L (crude extract). In the first fractionation procedure (a) in Figure 2, the crude extract is separated by any of several methods (e.g., chromatography) into several fractions (fractions 1, 2, 3, 4, , ,). After the removal of the solvents, the weight of each residue will be measured (B_1, B_2, B_3, B_4, , , kg). Ideally, the sum of the weights of all fractions (B_1, B_2, B_3, B_4, , , kg) is equal to A kg. Each

Downloaded by CORNELL UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch009

Downloaded by CORNELL UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch009

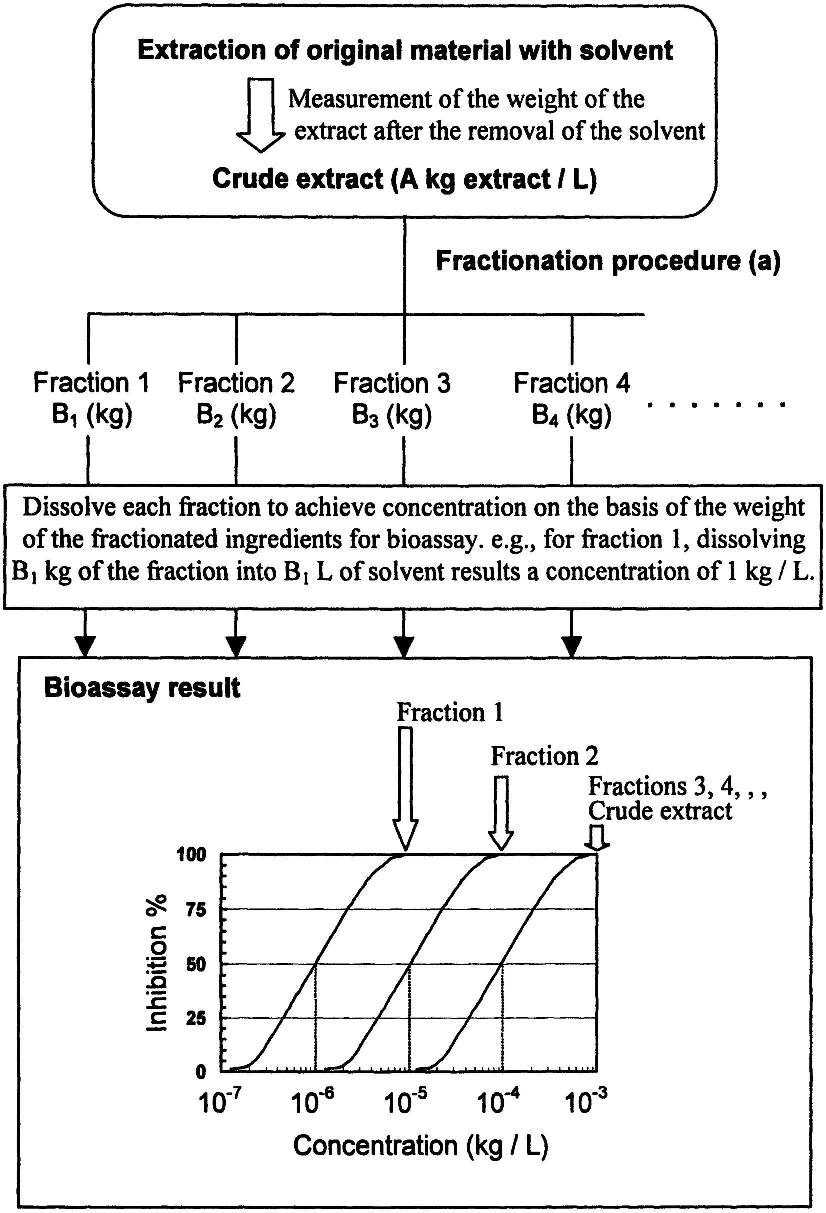

Extraction of original material with solvent
Measurement of the weight of the extract after the removal of the solvent
Crude extract (A kg extract / L)
Fractionation procedure (a)
Fraction 1
B_1 (kg)
Fraction 2
B_2 (kg)
Fraction 3
B_3 (kg)
Fraction 4
B_4 (kg)
Dissolve each fraction to achieve concentration on the basis of the weight of the fractionated ingredients for bioassay. e.g., for fraction 1, dissolving B_1 kg of the fraction into B_1 L of solvent results a concentration of 1 kg / L.
Bioassay result
Fraction 1
Fraction 2
Fractions 3, 4, , ,
Crude extract
100
75
50
25
0
Inhibition %
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
Concentration (kg / L)

Figure 2. A typical procedure to follow the specific activity as an isolation indicator. Symbols B_1, B_2, B_3, and B_4 represent the weight of the fractions 1, 2, 3, and 4, respectively. A concentration to induce 50% inhibition is decreased with increasing purity.

fraction will then be dissolved into a solvent to achieve a concentration on the basis of the weight of the fraction for bioassay. In case of fraction 1, for example, dissolving B_1 kg of the fractionated material into B_1 L of solvent results a concentration of 1 kg/L (10^6 ppm). A bioassay experiment will be conducted on the basis of this concentration. In the case of Figure 2, a concentration to induce 50% inhibition for the crude extract is 10^{-4} kg/L. For fractions 1, 2, 3, and 4, these concentrations are 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-4} kg/L, respectively. Because the crude extract contains impurities with no or lower inhibitory activity, a purified fraction containing the bioactive compounds should have higher inhibitory activity (lower concentration to induce half-maximum action). In the case of Figure 2, fraction 1 shows the highest inhibitory activity, and fractions 2, 3, and 4 are 10- to 100-fold weaker than fraction 1.

Of course, fraction 1 will be the most important candidate for further purification. In this case, concentration to induce 50% inhibition will decrease as purification progresses. However, it does not mean that the other fractions are of no use. The calculated activities are influenced by the amount of active compound and that of non-active impurities. When the crude extract contains some kinds of active compounds, it will be possible that fraction 2 contains a more active compound than fraction 1, although at a low concentration. To avoid this problem, it will be effective to produce more fractions to achieve higher purity of the active compounds, even if the yield of the active compounds is reduced.

It is also important to compare their activities at concentrations inducing half-maximum action. If comparison is made on the basis of maximum or minimum action, the activity level will be obscured. Therefore, the biological activities should be clarified at several concentrations to estimate the concentration which induces half-maximum action.

Isolation Procedure to Follow the Total Activity as an Indicator

To calculate a value of the total activity according to equation 1, the concentration and the specific activity of the compound should be known, although they are always unknown in the purification procedures. However, it is possible to calculate the total activity even for the crude extract. Remember that the concept of the total activity is "the dilution ratio to obtain EC_{50}." When 1 kg of the original organism is extracted and the volume of the extract is adjusted to 1 L (1 kg /1 L), the dilution ratio of the solution can be conventionally regarded as unity. A bioassay experiment of this solution, with a series of appropriate dilution ratios, will give a value of the total activity of this organism.

For example, assume that a crude extract is obtained by extracting A_1 kg of the original material (or organism) with A_2 L of solvent, resulting in a concentration of A_1 kg original material / A_2 L (Figure 3). This concentration

Downloaded by CORNELL UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch009

may be adjusted to desired level by condensation or dilution if preferred. At this stage, the weight of the extracted ingredients is not essential information for the calculation of the total activity, but the weight of the original material used for the extraction is essential information. In the case of Figure 3, bioassay experiments for several dilution ratios of the crude extract make it clear that the concentration to induce half-maximum action (inhibition) of the crude extract is 10^{-4} kg/L. Accordingly, the total activity (the dilution ratio to obtain EC_{50}) is calculated to be 10^4.

After fractionation of the crude extract by a fractionation procedure (a) in Figure 3, the volume of each fraction will be adjusted to A_2 L to obtain a concentration on the basis of the original material for bioassay, resulting a concentration of A_1 kg original material / A_2 L for each fraction. These fractionated solutions will then be subjected to bioassay on the basis of this concentration. In Figure 3, concentrations to induce 50% inhibition for fractions 1, 2, 3, and 4 are ca. 10^{-4}, 10^{-2}, 10^{-2}, and 10^{-2} kg/L, respectively. Inhibitory activity of fraction 1 is almost identical with that of the crude extract, indicating that almost the entire activity of the crude extract is recovered in fraction 1. The inhibitory activities of fractions 2, 3, and 4 are almost 100-fold weaker than that of the crude extract and fraction 1. This means that fraction 1 contains the active compound responsible for almost all of the activity of the crude extract, and fractions 2, 3, and 4 are not important because they explain only 1/100 of the total activity of the crude extract. In this comparison, both the specific activity and the concentration factors are taken into account.

In an isolation procedure where the total activity is an indicator, it is important to confirm that the activity is recovered at a high level, preferably more than 50%, to make sure that the major part of the activity is recovered by the purification procedure. In the case of Figure 3, almost all of the activity of the crude extract is recovered in fraction 1. However, if the recovery of the activity is much less than 50%, it might be possible that the most important compound is lost in the purification procedures. In such cases, the fractionation procedure is not suitable to follow the total activity. To achieve a high yield of the activity, the target compound should be fractionated into one fraction, even though it might be contaminated with some impurities. This point differs with the isolation procedure where the specific activity is the main concern, and in this case purity is more important than the recovery.

As described above, the isolation strategy is the key to select the purification procedure. It also determines the meaning of the purified compounds. Therefore, the selected isolation strategy should be declared in the scientific discussion, although this is not always mentioned. Here, the author proposes the use of the terminologies and concepts of "specific activity" and "total activity" to clarify the differences between them and to avoid confusion in scientific discussions.

Downloaded by CORNELL UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch009

Downloaded by CORNELL UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch009

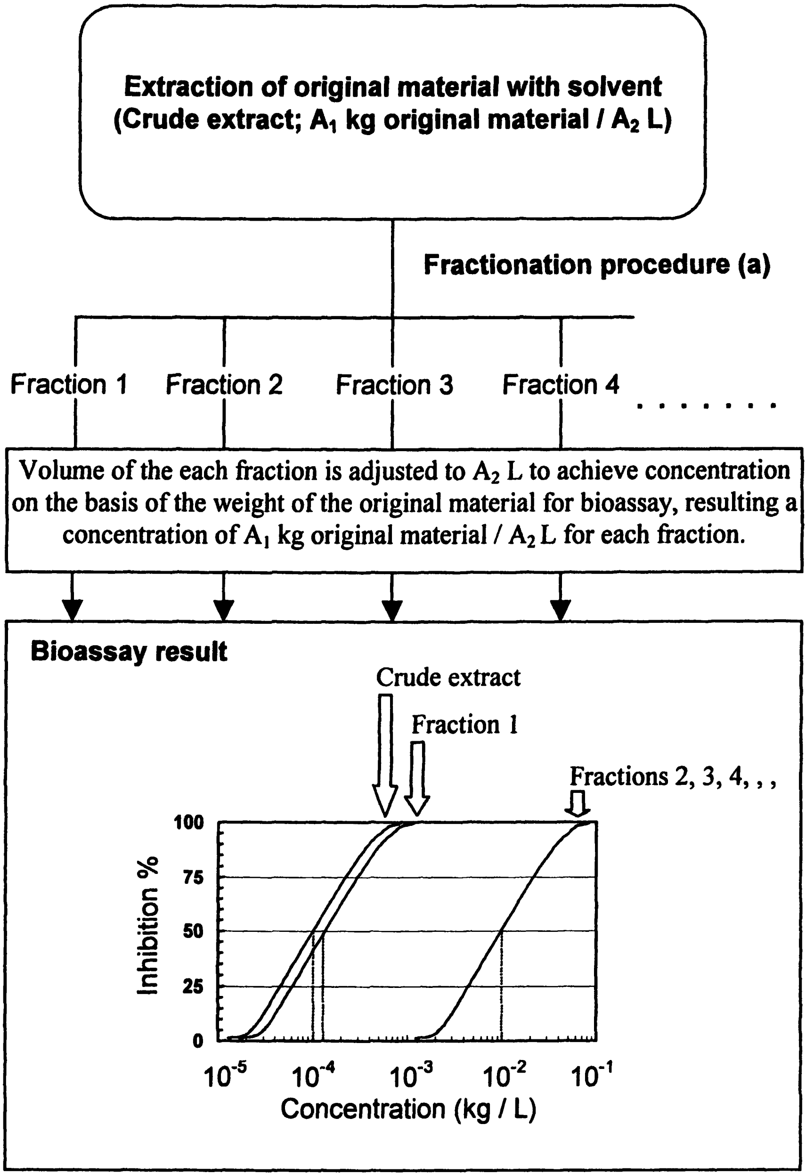

Extraction of original material with solvent
(Crude extract; A_1 kg original material / A_2 L)
Fractionation procedure (a)
Fraction 1
Fraction 2
Fraction 3
Fraction 4
. . . .
Volume of the each fraction is adjusted to A_2 L to achieve concentration on the basis of the weight of the original material for bioassay, resulting a concentration of A_1 kg original material / A_2 L for each fraction.
Bioassay result
Crude extract
Fraction 1
Fractions 2, 3, 4, , ,
Inhibition %
100
75
50
25
0
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
Concentration (kg / L)

Figure 3. A typical procedure to follow the total activity as an isolation indicator. When the recovery is high enough in the fractionation procedure (a), the concentration to induce 50% inhibition should be kept almost constant in the active fraction (Fraction 1).

Specific and Total Activities in Literature

Application of the specific activity approach is common not only in searching for pesticides and their prototypes but also in ecological research. For ecological research, however, the specific activity strategy does not always result in isolation of the most important compounds. In this section, some findings on allelopathy are reviewed with special reference to the specific and total activities.

Specific and Total Activities of Reported Allelochemicals

Chemical interactions among plants through bioactive compounds are categorized as allelopathy, and the bioactive chemicals are referred as allelochemicals. To isolate allelochemicals, the use of the total activity would be helpful, because it could reveal the most influential compound in the organism that would be involved in its chemical ecology interactions, including allelopathy. After identifying the possible allelochemicals, the values of the total activity could explain the potential role and influence of the compounds on allelopathy. It would also be valuable to calculate the total activity for the explanation of the autotoxicity. On the other hand, the specific activity clarifies the biological activity of the compound itself. In literature, many allelochemicals have been reported, although their specific activities and total activities are seldom reported. Here, attempts are made to re-evaluate plant growth inhibiting allelochemicals using both the specific and total activities (Table I).

Plant growth inhibition under the canopy of black walnut (*Juglans nigra*) is one of the most well-known allelopathic phenomenon, and it has been proposed that this phenomenon is caused by juglone and its glucoside (juglone potential, Figure 4), although proof of this explanation is still being debated. The specific activity of juglone was estimated to be 10^{-5} *M* level, and the total activity of juglone potential in the leaf of black walnut ranged between 25 to 75, depending on the growing season. In the case of (+)-2-*cis*-4-*trans*-abscicis acid (ABA), which is a phytohormone found in all plants, the specific and total activities were estimated to be 3×10^{-6} *M* and 0.07 to 0.4, respectively. The specific activity of (+)-2-*cis*-4-*trans*-ABA would be almost 3-fold higher than that of the juglone potential, and its plant growth inhibitory activity could be in the highest level among natural products. However, its total activity is 60- to 1100-fold lower than that of the juglone potential because the amount of (+)-2-*cis*-4-*trans*-ABA present in plants is much smaller than that of juglone and its glucoside, indicating that juglone in black walnut would be more influential than (+)-2-*cis*-4-*trans*-ABA.

Similar comparisons are also possible among other allelochemical candidates as listed in Table I. Momilactone B, 2,4-dihydroxy-7-methoxy-1,4- benzoxazin-3-one (DIMBOA), and 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) are also reported as possible allelochemicals, but their total activities are lower than that of the juglone potential. On the other hand, L-3,4-dihydroxyphenylalanine (L-

Downloaded by CORNELL UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch009

Table I. Inhibitory Activity of Possible Allelochemicals Expressed as Specific Activity and Total Activity

Donor Plant (Origin)	*Compound*[a]	*Specific Activity* (*M*)	*Total Activity*[b] (no unit)	*Acceptor Plant (reference)*
Oryza sativa	Momilactone B	10^{-4}	0.01 - 0.1	Lettuce (*1, 2*)
Brassica oleracea	(+)-2-*cis*-4-*trans*-ABA	3×10^{-6}	0.07	Rice (*3, 4*)
Rosa canina	(+)-2-*cis*-4-*trans*-ABA	3×10^{-6}	0.4	Rice (*3, 4*)
Agropyron repens	DIMBOA	7×10^{-4}	0.1	Wild Oat (*5,6*)
	DIBOA	4×10^{-4}	7	Cress (*5, 7*)
Vicia villosa	Cyanamide	3×10^{-4}	4 - 35	Lettuce (*8*)
Juglans nigra	Juglone and its glycoside	10^{-5}	25 - 75	Crimson Clover (*9, 10*)
Solidago altissima	*cis*-DME	3×10^{-5}	40 - 80	Rice (*11, 12*)
Mucuna pruriens	L-DOPA	2×10^{-4}	200	Lettuce (*13*)
Duranta repens	Durantanins I, II, and III	5×10^{-5}	200	Lettuce (*14*)
Spiraea thunbergii	*cis*-CG and *cis*-BCG	3×10^{-6}	1000	Lettuce (*15*)

[a] Chemical structures are shown in Figure 4.

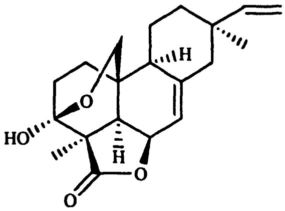
[b] Estimated by equation 1. See text.

Downloaded by CORNELL UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch009

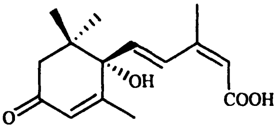
DOPA), durantanins I to III, and *cis*-CG plus *cis*-BCG have higher total activities than the juglone potential.

The total activity would not always be related to the allelopathic activity in the ecosystems, because of the possibility such that some allelochemical candidates are inactivated in soils, or the release of these compounds from the organisms is restricted. Nevertheless, the total activity would be a useful indicator to reveal "allelopathic potentials." Calculation of the total activity using equation 1 would enable the comparison of the allelopathic potentials of organisms or extracts.

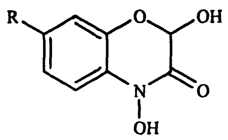
Downloaded by CORNELL UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch009



Momilactone B



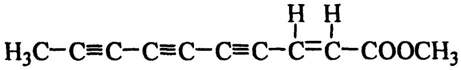
(+)-2-*cis*-4-*trans*-Abscisic acid (ABA)



DIMBOA, R = OCH_3
DIBOA, R = H

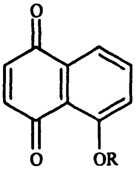
$H_2N{-}C{\equiv}N$

Cyanamide

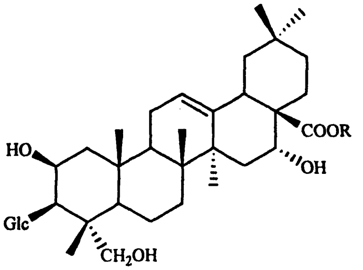


$$H_3C{-}C{\equiv}C{-}C{\equiv}C{-}C{\equiv}C{-}\overset{H}{C}{=}\overset{H}{C}{-}COOCH_3$$

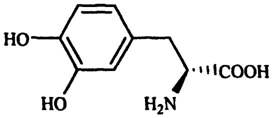
cis-Dehydromatricaria ester (*cis*-DME)



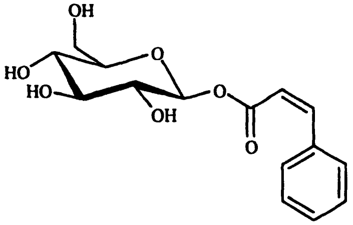
R = H; Juglone
R = Glc; Glucosyl juglone



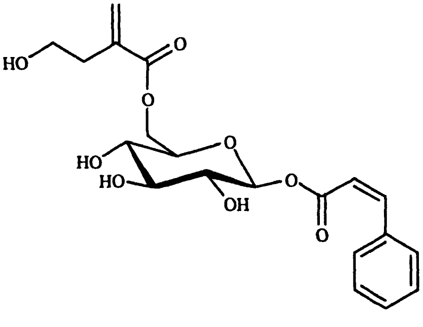
R = Rha-Api-Rha-Ara; Durantanin I
R = Rha-Xyl-[Api]-Rha-Ara; Durantanin II
R = Rha-Xyl-Rha-Ara; Durantanin III



L-3,4-Dihydroxyphenylalanine (L-DOPA)



1-*O*-*cis*-Cinnamoyl-ß-D glucopyranose (*cis*-CG)



6-*O*-(4'-Hydroxy-2'-methylene-butyroyl)-1-*O*-*cis*-cinnamoyl-ß-D-glucopyranose (*cis*-BCG)

Figure 4. Chemical structures of possible allelochemicals. DIMBOA: 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one DIBOA: 2,4-Dihydroxy-1,4-benzoxazin-3-one.

cis-Dehydromatricaria Ester (*cis*-DME) from *Solidago altissima*

Solidago altissima (Compositae) has been reported to show an allelopathic phenomenon and to contain *cis*-dehydromatricaria ester (*cis*-DME) as a possible allelochemical (*11*). Although the specific activity strategy was applied in the isolation procedure in finding *cis*-DME, Kobayashi et al. (*11*) proposed significant allelopathic role of *cis*-DME in the ecosystem from the evidences of its high specific activity, high contents in the plants and soils, and significant effects of soil-applied *cis*-DME on the growth of pot-cultured plants (*Ambrosia artemisiaefolia*). The allelopathy of *S. altissima* caused by *cis*-DME, however, has been questioned because the concentration of *cis*-DME in soil solution in a volcanic ash soil is much lower than the influential level (*12*). This result is quite understandable, since the solubility of *cis*-DME in water is very low. However, the allelopathic potential of *S. altissima* through *cis*-DME could not be totally denied, because the concentration of *cis*-DME in soil solution would depend on the type of soils. Furthermore, it would be possible that a very poorly soluble compound acts as an active ingredient in the release-control interaction between the compound and soils, like in the case of synthetic herbicides in soils (e.g., trifluralin). Therefore, allelopathy of *S. altissima* caused by *cis*-DME remains to be debated. Here, it should be remembered that there is no evidence showing that *cis*-DME is the most influential compound in the original plant extract of *S. altissima* because *cis*-DME was found under the specific activity strategy. Although the total activity of *cis*-DME in *S. altissima* (40 to 80) is at a relatively high level (same level as juglone potential of *J. nigra*, Table I), it would therefore be valuable to reassess the crude extract of *S. altissima* following the total activity as an indicator. Our preliminary experiment showed that rhizosphere soil of *S. altissima* also inhibited the growth of lettuce seedlings (data not shown). Therefore, it would also be important to isolate plant growth inhibiting compounds from the rhizosphere soils following the total activity strategy.

Downloaded by CORNELL UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch009

Plant Growth Inhibiting Compounds in *Spiraea thunbergii*

High plant growth inhibitory activity has been reported in the leachate of an ornamental plant, *Spiraea thunbergii* (Rosaceae). To isolate the causal compounds, bioassay-directed fractionation was conducted following the total activity as an isolation indicator, resulting into the identification of two causal compounds, 1-*O*-*cis*-cinnamoyl-*β*-D-glucopyranose (*cis*-CG, Figure 4) and 6-*O*-(4'-hydroxy-2'-methylene-butyroyl)-1-*O*-*cis*-cinnamoyl-*β*-D-glucopyranose (*cis*-BCG, Figure 4) (*15*). Although the plant growth inhibitory activity of the crude extract was divided into two different fractions (water and ethyl acetate fractions) at the first fractionation procedure (liquid-liquid partition), the chemical structures of the compounds obtained from the two fractions were close to each other. It is also notable that the specific activities of *cis*-CG and *cis*-BCG are at the highest level among natural products, which are comparable with that of (+)-2-*cis*-4-*trans*-ABA (Table I). In this case, therefore, the total activity strategy yielded causal compounds with high specific activities. If the specific activity was taken as an isolation indicator, only *cis*-BCG might be isolated and *cis*-CG could be missed, because *cis*-CG was fractionated into the water fraction in the first fractionation procedure with large amount of impurities and the content of *cis*-CG in *S. thunbergii* was smaller than that of *cis*-BCG, resulting in extremely lower specific activity in the water fraction than in the ethyl acetate fraction.

Downloaded by CORNELL UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch009

Conclusion

Isolation strategies are summarized using two terms: specific activity and total activity. Concepts and application methods of the two isolation strategies are described with some research examples. The author has proposed to use the terminologies and concepts of “total activity” and “specific activity” to clarify scientific discussions of bioactive natural compounds in the contexts of chemical ecology and compounds for use as pesticides. The total activity and the specific activity will be more important in chemical ecology and in searching highly active pesticides, respectively.

References

1. Lee, C. W.; Yoneyama, K.; Takeuchi, Y.; Konnai, M. *Biosci. Biotechnol. Biochem.* **1999,** *63,* 1318.
2. Kato-Noguchi, H.; Ino, T.; Sata, N.; Yamamura, S. *Physiol. Plant.* **2002,** *115,* 401.
3. Koshimizu, K.; Fukui, H.; Mitui, T.; Ogawa, Y. *Agr. Biol. Chem.* **1966,** *30,* 941.
4. *Biologically Active Natural Products: 2nd Ed.;* Takahashi, N.; Marumo, S.; Otake, N., Eds.; Univ. Tokyo Press: Tokyo, Japan, 1981; p 65. (*In Japanese*)
5. Friebe, A.; Schulz, M.; Kück, P.; Schnabl, H. *Phytochemistry* **1995,** *38,* 1157.
6. Pérez, F. J. *Phytochemistry* **1990,** *29,* 773.

7. Barnes, J. P.; Putnam, A. R.; Burke, B. A.; Aasen, A. J. *Phytochemistry* **1987,** *26,* 1385.
8. Kamo, T.; Hiradate, S.; Fujii, Y. J. *J. Chem. Ecol.* **2003,** *29,* 275.
9. Rietveld, E. J. *J. Chem. Ecol.* **1983,** *9,* 295.
10. Coder, K. D. *J. Chem. Ecol.* **1983,** *9,* 1203.
11. Kobayashi, A.; Morimoto, S.; Shibata, Y.; Yamashita, K.; Numata, M. *J. Chem. Ecol.* **1980,** *6,* 119.
12. Ito, I.; Kobayashi, K.; Yoneyama, T. *Ann. Bot.* **1998,** *82,* 625.
13. *Biologically Active Natural Products: Agrochemicals;* Cutler, H. G.; Cutler, S. J., Eds.; CRC Press: Boca Raton, FL, 1999; p 33.
14. Hiradate, S.; Yada, H.; Ishii, T.; Nakajima, N.; Ohnishi-Kameyama, M.; Sugie, H.; Zungsontiporn, S.; Fujii, Y. *Phytochemistry* **1999,** *52,* 1223.
15. Hiradate, S.; Morita, S.; Sugie, H.; Fujii, Y.; Harada, J. *Phytochemistry* **2004,** *65,* 731.

Downloaded by CORNELL UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch009

Downloaded by UNIV OF GUELPH LIBRARY on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch010

Chapter 10

Natural Products and Their Role in the Design of Active Ingredients for Modern Crop Protection

Peter Jeschke, Folker Lieb, Robert Velten, and Welf-Burkhard Wiese

Bayer CropScience AG, BCS-R-GC1, Building 6240, Alfred-Nobel-Strasse 50, D-40789 Monheim am Rhein, Germany

Today only a few natural products are directly used as active ingredients within the current main areas of modern crop protection. However, the influence of synthetic commercial agrochemicals having a natural origin is remarkable for the total insecticide and fungicide market. The successful utilization of natural products and their role as lead structures in the design of synthetic analogues as active ingredients is exemplified with commercial products from Bayer Crop Science. The molecular diversity of natural products plays a dominant role in the discovery process and has opened up new approaches for lead-finding as well. The dereplication process, novel and complementary technologies and screening methods for a high number of compounds are a powerful tool in the search for hitherto unknown compounds.

In Natural Products for Pest Management; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

Downloaded by UNIV OF GUELPH LIBRARY on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch010

Why should the agrochemical industry be interested in natural products (NPs) as a potential source of new active ingredients (a.i.s) for modern crop protection? One answer could be, that NPs, even those having no agricultural relevance, have substantially contributed to the identification and understanding of novel biochemical pathways. A legendary example is the elucidation of the mode of action (MoA) of the alkaloid physostigmine which makes an important contribution to the clarification of the neurophysiology and biochemistry of acetylcholine.

NPs can be used either directly in modern crop protection or can serve as lead structures for the development of new synthetic analogues with favourable biological and physicochemical properties. A survey of commercial products shows that NPs account only for around 3 % *(1)* (Figure 1).

88.7 %	Synthetic Products
5.6 %	Biologicals
3.3 %	**Natural Products**
2.4 %	Semiochemicals

⇨

40.8 %	**Insecticides / Acaricides**
22.2 %	**Fungicides**
18.5 %	**Plant Growth Regulators**
11.1 %	**Rodenticides**
7.4 %	**Herbicides**

Figure 1. Differentiation of NPs outlined in "The Pesticide Manual" (1)

Today only a few NPs are directly used as a.i.s in crop protection. For direct use in agriculture, a NP should be:

- Sufficiently efficacious against target species like insects, fungi or weeds.
- Safe and biologically selective with a good crop selectivity.
- Standardized for composition and formulation.
- Readily available, *e.g.* by synthesis, extraction or fermentation processes.

If these criteria are not met, appropriate structural modifications might lead to a marketable product. Because of their complex structures, most commercial NPs have to be prepared by:

1. Fermentation processes, *e.g.* *(i)* the fungicides blasticidin-S (Bla-A®), kasugamycin (Kasugamin®) and mildiomycin (Milanesin®); *(ii)* the insecticide and acaricide abamectin (Agrimec®, Vertimec®); *(iii)* the insecticides milbemectin (Milbeknock®, Mesa®) and spinosad (Tracer®, Success®); *(iv)* the herbicide bilanaphos, bialaphos (Meiji Herbiacae®).

2. Extraction processes, *e.g.* the insecticides pyrethrum (Alfadex®, Evergreen®) and rotenone (Noxfire®).

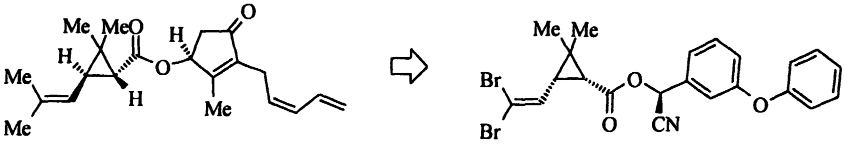
Only the insecticide emamectin benzoate is a structural modification of the fermentation product abamectin, produced by *Streptomyces avermitilis*.

Downloaded by UNIV OF GUELPH LIBRARY on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch010

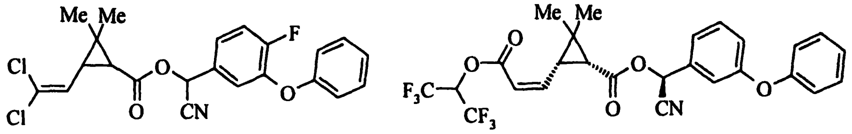
Products from Bayer CropScience with a Natural Origin

The successful use of NPs and their innovative potential as lead structures in design of simple synthetic analogues as a.i.s for modern crop protection can be exemplified with selected commercial products from Bayer CropScience, like:

(a) Insecticidal pyrethroids: The development of synthetic pyrethroids, which act on the voltage gated sodium channel, provides a significant historical illustration of a.i.s regarding simplification of the natural pyrethrins. Shortening and modifying the pentadienyl side chain of pyrethrin I 1 *(2)*, led in the 1950s to the first synthetic and more stable pyrethroid allethrin *(3)*. More than 20 years later, a systematic structural modification gave various photostable synthetic products with a wide range of agricultural applications. The so-called *"evolution"* of pyrethroids can be demonstrated by three selected examples, having only little structural similarity with the natural origin 1 (Figure 2).



1: Pyrethrin I (Pyrethrum) **2**: Deltamethrin, 1977 (Roussel-Uclaf)



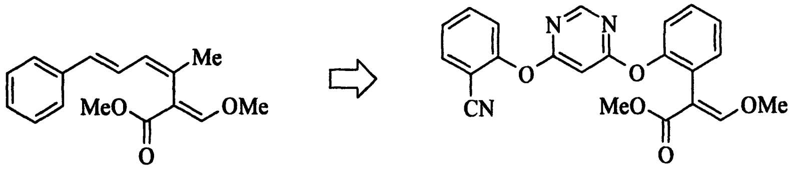
3: beta-Cyfluthrin, 1980 (Bayer CropScience) **4**: Acrinathrin, 1991 (Roussel-Uclaf)

Figure 2. Structural evolution of insecticidal pyrethroids from pyrethrin I 1

Replacement of the cyclopentene alcohol group, introduction of the Br_2C=CH- moiety, incorporation of the phenoxy-benzyl alcohol and insertion of a α-CN in the benzyl-CH_2 group produced deltamethrin **2** (Decis®) *(4)*, which shows a significant enhancement in insecticidal activity. Beta-Cyfluthrin **3** (Bulldock®) *(5)* is a successful example for the effect of fluorine substitution in the already outstandingly active cypermethrin *(6)*. Finally, replacing the terminal vinyl halogen by a fluorine-containing ester group led to acrinathrin **4** (Ardent®) *(7)*, which was commercialized as an insecticide and acaricide.

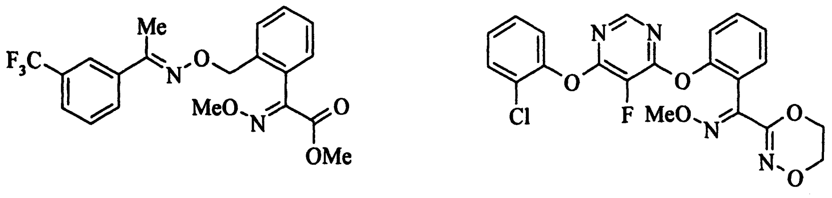
(b) Fungicidal β-methoxyacrylates: The discovery of strobilurins, an important class of agricultural fungicides, was inspired by a group of natural fungicidal

derivatives of β-methoxyacrylates, the simplest of which are strobilurin A **5** *(8)*, oudemansin A *(9)* and myxothiazol A *(10)*. All stobilurins inhibit mitochondrial respiration chain by binding at the Q_o site of cytochrome b *(11)*. Systematic modification of the light-sensitive polyenic structure in **5** led to the stabilized enol ether stilbene, and finally to the first marketable fungicide azoxystrobin **6** *(12)* from Zeneca (today Syngenta) (Figure 3).



5: Strobilurin A

6: Azoxystrobin, 1996 (ICI, Zeneca)



7: Trifloxystrobin, 1999 (Novartis)

8: Fluoxastrobin, (Bayer CropScience)

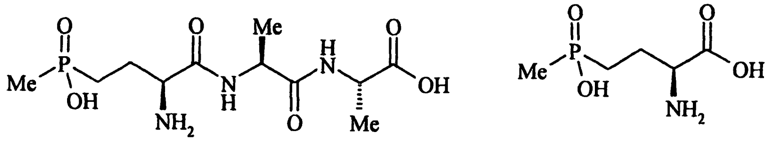
Figure 3. Structural evolution of β-methoxyacrylates from strobilurin A 5

With trifloxystrobin **7** (Flint®) *(13)* - Bayer's acquisition from Syngenta in 2000 - the company Bayer made its first step into this new generation of strobilurin fungicides. The latest example is fluoxastrobin **8** (HEC™) *(14)*. This novel leaf systemic (penetration and translocation into plant leaves) broad spectrum fungicide of dihydrodioxazine type is currently being developed by Bayer CropScience mainly for use in cereal crops. It provides both a rapid initial effect and prolonged activity due to its protective and leaf systemic properties. Applied as a foliar spray in cereals, **8** provides excellent control of *e.g. Septoria* leaf spot and glume blotch, rust, *Helminthosporium* diseases in wheat and barley, as well as in *Rhychosporium secalis* and *Blumeria graminis* spp..

(c) Herbicidal phosphinic acids: Bilanafos **9** (or bialaphos) isolated from culture broth of *Streptomyces* strains is active against broad-leaved weeds. The pro-herbicide **9** is a tripeptide with two alanyl units that is metabolically converted into *(S)*-glufosinate **10** (or phosphinotricin) within the plant by hydrolytic cleavage. This unique methyl phosphinic acid analogue of glutamic acid is a strong competitive inhibitor of the enzyme glutamine synthase. Examination of

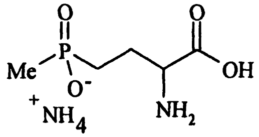
Downloaded by UNIV OF GUELPH LIBRARY on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch010

the herbicidal effect of **10** after its metabolic breakdown led to glufosinate-ammonium **11** (Basta®) which is manufactured and used as a racemate for controlling a wide range of annual and perennial broad-leaved weeds (Figure 4).



9: Bilanafos, 1984 (Meiji Seika)

***(S)*-10**: *(S)*-Glufosinate



***rac*-11**: Glufosinate-ammonium, 1986 (Hoechst)

Figure 4. Structural evolution of herbicides from natural bilanafos 9

To broaden the application range of **11** as a herbicide, the gene for the corresponding *N*-acetyltransferase was successfully transferred to plants in order to generate Basta-resistant transgenic crops - like Liberty Link™.

Influence of Natural Products in Design of Synthetic Active Ingredients

The share of synthetic commercial agrochemicals having a natural origin in the total insecticide and fungicide market is remarkable, as demonstrated especially for the structural evolution of synthetic pyrethroids and of strobilurins.

In 2002, the global crop protection market was valued at 26,610 €M. Whereas the market share of synthetic herbicides, resulting directly from **9**, accounts for around 1 %, the fungicide market share of synthetic strobilurins accounts for around 13 % (761 €M). Finally, the insecticide market share of synthetic pyrethroids accounts for nearly 16 % (1,064 €M). Today the pyrethroids represent the largest group of NP-derived insecticides. The latest *The Pesticide Manual (1)* lists 40 synthetic compounds within this chemical class.

Search for New Sources of Natural Products

Since the 1980s, the search of novel sources for pesticides within Bayer CropScience includes NPs from bacteria and eukarya-like plants and fungi. Several examples based on plants like *Crinum powellii*, *Aglaia* spp., *Annona squamosa* and *Taccaceae*; fungi like *Omphalotus olearius*, *Fusarium* spp.,

Downloaded by UNIV OF GUELPH LIBRARY on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch010

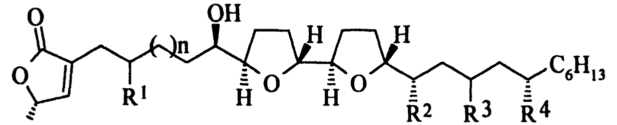
Amblyosporium and *Pyrenochaeta terrestris*, as well as bacteria like *Nocardia* spp., may be potential sources of agricultural relevant compounds for major agrochemical areas, like:

- Insecticides, *e.g.* annonins, cripowellin and rocaglamide.
- Nematicides, *e.g.* omphalotin A and taccalonolides.
- Herbicides, *e.g.* pyrenocines and thiolactomycin.
- Fungicides, *e.g.* enniatins and amblyomycin.

Insecticidal Active Ingredients

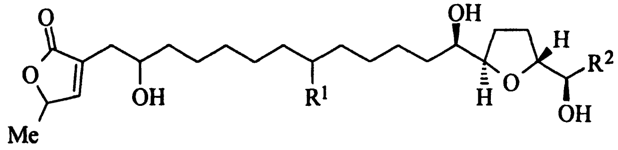
In the late 1980s, Bayer started working in the area of NPs. Plant extracts were screened, and the a.i.s were isolated to identify novel biologically active compounds.

(a) Annonins: Several annonins, like the cytotoxic annonine I **12**, were isolated from 120 kg ground seeds of *Annona squamosa* by a double extraction procedure and subsequent purification *(15)* (Figure 5).



type A

12: Annonin I $R^1 = H, R^2 = $ ''''OH, $R_3 = OH, R^4 = H, n = 9$



type B

13: Annonacin A $R^1 = OH, R^2 = C_{12}H_{25}$

14: Annonastatin $R^1 = H, R^2 = C_{15}H_{31}$

Figure 5. Structure of isolated annonins (12-14)

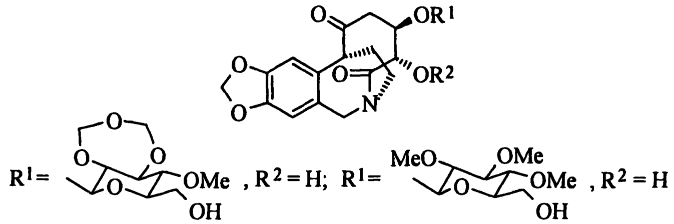
Compound **12** is an inhibitior of the mitochondrial respiratory chain (complex I). Besides type A annonins, containing two tetrahydrofuryl rings close together, type B derivatives, containing only one tetrahydrofuryl ring, were isolated. Type A annonins mainly differ from each other with respect to their stereochemistry, the degree of hydroxylation and the chain lengths. Type B,

Downloaded by UNIV OF GUELPH LIBRARY on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch010

represented by annonacin A **13** and annonastatin **14**, show a similar differentiation. Type A is the most insecticidally active compound. Structure activity relationships (SARs) for the isolated annonins show that type A is more potent than type B because:

- Three hydroxy groups are essential for the insecticidal activity.
- The precise positions of the hydroxy groups is crucial.
- The distance between the tetrahydrofuryl rings and butenolide is important.
- Two tetrahydrofuryl rings increase activity.

(b) Cripowellins: Extracts of the *Amaryllidaceae* plant *Crinum powellii* show strong insecticidal activity. After extraction of 450 kg of fresh bulbs and chromatographic purification, two novel insecticidally active alkaloids - cripowellin A **15a** and B **15b** were isolated and their structures were elucidated *(16)* (Figure 6).



15a: Cripowellin A

16: $R^1 = H$; $R^2 = COMe$

15b: Cripowellin B

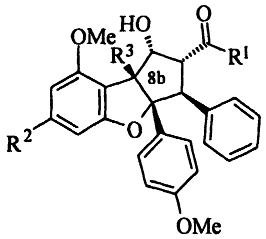
Figure 6. Cripowellin A ***15a****, B* ***15b*** *and the aglycone* ***16***

In order to get more detailed information about the role of the sugar moiety biological activity, hydrolysis to the aglycone **16** was carried out. Surprisingly, **16** is as active as **15a** against insects. A comparison of insecticidal and acaricidal activity for both **15a** and **b** has demonstrated that **15a** is more potent than **15b**, both against coleopteran pests (*e.g., Phaedon cochleariae*) and homopteran pests (*e.g., Myzus persicae*). But **15b** showed better activity against lepidopteran pests (*e.g., Spodoptera frugiperda*). On the other hand, both **15a** and **b** are equally active against *Plutella xylostella* and against the spider mite *Tetranychus urticae.*

(c) Rocaglamide: **17** and its derivatives, isolated from *Aglaia species,* exhibit antileukemic activity *(17)* as well as insecticidal activity comparable to that of azadirachtin. In order to identify insecticidally active derivatives, a facile access to the cyclopentabenzofuran core structure was developed *(18)* (Figure 7).

Downloaded by UNIV OF GUELPH LIBRARY on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch010

However, compound **18** has only weak insecticidal activity against *P. cochleariae* and *S. frugiperda* at 1000 ppm.



17: R^1 = NMe , R^2 = OMe, R^3 = OH Rocaglamide
18: R^1 = OEt, R^2, R^3 = H

Figure 7. Rocaglamide 17 and its analogue 18

Because analogues of **17** with different aryl substitution patterns are active, one can assume that the difference in position C8b - hydrogen instead of hydroxy - is important for the insecticidal activity.

Nematicidal Active Ingredients

(a) Omphalotin A: This cyclic dodecapeptide **19** produced by submerged cultures of the basidiomycete *Omphalotus olearius*, exhibits nematicidal activity *(19)*. The root-knot nematode *Meloidogyne incognita* was the most sensitive. Incorporated into agar, **19** at 1 mg/L prevents infection of cucumber seedlings by *M. incognita*. On the other hand, in greenhouse studies **19** at doses between 2.5 and 10 mg/L provided complete protection of cucumber and lettuce against *Radopholus similis*, *Heterodera schachtii* and *Pratylenchus penetrans*.
Larger amounts of **19** for screening were prepared by solid-phase (SP) peptide synthesis (Prof. Jung, Univ. Tübingen) *(20)* (Figure 8).

Fmoc-MeVal-MeIle-Sar-Val-MeIle-Sar-Trp-MeVal-Ile-MeVal-MeVal-Sar-**R**

↓
1. Fmoc cleavage
2. cleavage from resin (R)
3. cyclization

Cyclo(Val-MeIle-Sar-Val-MeIle-Sar-Trp-MeVal-Ile-MeVal-MeVal-Sar)

19: Omphalotin A

Figure 8. Structure and amino acid sequence of omphalotin A 19

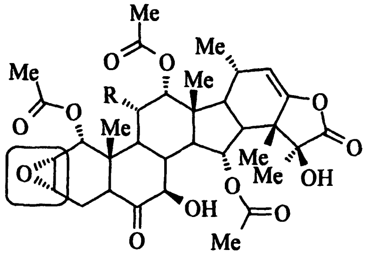
The racemization-free peptide coupling of Fmoc-protected amino acids on the SP with a trityl anchor (R = TCP-resin) to form the precursor is achieved with a

Downloaded by UNIV OF GUELPH LIBRARY on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch010

triphosgene-activation method *(21)*. After cleavage of the Fmoc group and finally cleavage from the SP with hexafluoroisopropanol, the cyclization of the open-chained precursor led to **19** in 31 % yield. With this method, the preparation of further derivatives useful for investigation of SAR was also possible *(22)*.

(b) Taccalonolides: The genus *Tacca* includes some 50 species distributed predominantly in tropical zones, especially in asiatic countries. Taccalonolide A **20a** is the first microtubule-stabilizing agent isolated from a plant and the first NP steroid identified to have these cellular effects *(23)*.

On the other hand, our biological assays gave first hints of nematicidal activity. Therefore 750 g of *Tacca paxiana* roots were extracted and the crude extract was separated by liquid-liquid partition. After chromatographic purification of the ethyl acetate fraction, two taccalonolides, A **20a** and E **20b**, were isolated in a yield of 234 mg. In order to get larger amounts of taccalonolides, a large scale isolation from about 1000 kg of *T. paxiana* roots was carried out; from 43 kg of crude extract around 30 g of the **20a,b** were obtained (Figure 9).



20a: R = O-COMe Taccalonolide A
20b: R = H Taccalonolide E

*Figure 9. Taccalonolide A **20a** and E **20b***

The nematicidal activity of the individual taccalonolides, **20a,b**, as well as of the mixture of both was confirmed. The root-knot nematode *M. incognita* was the most sensitive. In greenhouse tests, complete reduction of root galls was achieved at 20 mg/L.

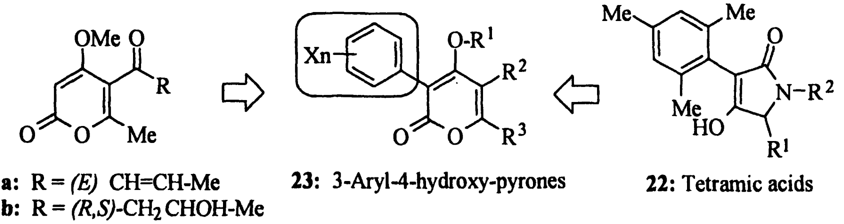
SAR studies revealed that the epoxide ring is not an essential structural element for this biological efficacy.

Herbicidal Active Ingredients

(a) Pyrenocines: Phytotoxins have the potential to serve as structural leads for herbicides. Pyrenocine A **21a** and B **21b** are two phytotoxins, produced by the phytopathogenic fungus *Pyrenochaeta terrestris*, which causes pink root disease in onions. The idea was to use both phytotoxins **21a,b** on the one hand and the herbicidally active tetramic acids **22** on the other hand, which are potent on

Downloaded by UNIV OF GUELPH LIBRARY on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch010

grasses in post- and pre-emergence treatment, as lead structures for the development of novel *"hybrid"* 3-aryl-4-hydroxy-pyrone herbicides **23** (Figure 10).



21a: R = *(E)* CH=CH-Me
21b: R = *(R,S)*-CH_2CHOH-Me

23: 3-Aryl-4-hydroxy-pyrones

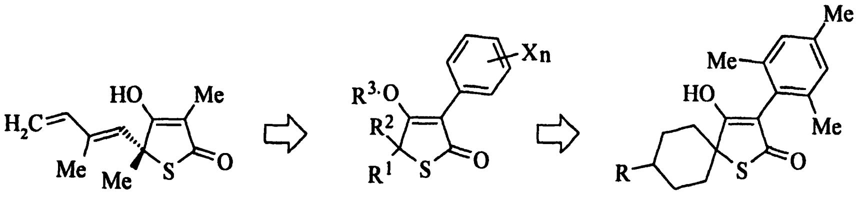
22: Tetramic acids

Figure 10. Pyrenocine A 21a and 21b as lead structures for new herbicides

A large number of different substituted 3-aryl-4-hydroxy-pyrones **23** was obtained in very good yields. Therefore, broad structural variations of **23** were possible.

It was found that the 3-aryl-4-hydroxy-pyrones **23** are inhibitors of acetyl-CoA-carboxylase (ACCase) *(24)* with pI_{50}-values between 4.4 and 5.8 (pI_{50}-values (= -logM) correspond to the concentration of cold ligand displacing 50 % of bound tritium-labeled ligand from ACCase). The mesityl group (phenyl with Xn = 2,4,6-Me_3) was identified as being the most important moiety in the 3-position. The pyrones **23** exhibit herbicidal activity against various weed species in post- and pre-emergence treatment.

(b) Thiolactomycin: The antibiotic thiolactomycin **24**, produced by the soil actinomycete *Nocardia spp.*, inhibits *de novo* fatty acid biosynthesis in higher plants. However, the *in vivo* activity is weak. In order to optimize this activity and to reduce the application rate, the 2(5*H*)thiophenone core with ketoenol functionality similar to the above mentioned pyrenocine A **21a** and herbicidally active tetramic acids **22**, served as a lead structure for novel tetronic acids **25** with enhanced herbicidal activity (Figure 11).



24: Thiolactomycin

25

26a: R = OMe
26b: R = H

Figure 11. Thiolactomycin 24 as herbicidal lead structure for 25 and 26

Downloaded by UNIV OF GUELPH LIBRARY on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch010

The 3-aryl-thiotetronic acid **26** containing the mesityl moiety has high intrinsic activity against ACCase (pI_{50}-values: **26a**: 5.5 and **26b**: 6.4), and good *in-vivo* herbicidal activity at 500 g/ha.

The 3-aryl-thiotetronic acids **26** are effective inhibitors of ACCase, similar to DMIs and FOPs, but at another binding site.

As already known, **26** blocks the acetoacetyl-acyl carrier protein (ACP). Other known herbicides, like DMIs of the cyclohexanedione type and grass herbicides of the diphenoxypropionic acid type (so-called FOPs) act on an earlier stage in the fatty acid biosynthesis.

Fungicidal Active Ingredients

a) Enniatins: A number of *N*-methylated cyclohexadepsipeptides (CHDPs), so-called enniatins such as enniatin A1, B or B1, have been isolated from different strains of *Fusarium*. Several biological activities of the CHDPs, like antibiotic, antifungal or phytotoxic activities, have already been published. The fungicidal activity is also comparable with *in-house* standards, *e.g.* enniatin B shows activity against plant pathogens and diseases, like the ascomycete *Botrytis cinerea*. In the 1990s it was found that naturally occuring enniatins also have antiparasitic activity *(25, 26)*.

In order to optimize the anthelmintic activity of naturally occuring enniatins, numerous DHDPs were prepared by total synthesis and macrocyclization of the appropriate linear hexadepsipeptide precursors. Replacement of *N*-methyl-isoleucine (MeIle) in enniatin A1 **27a** (Figure 12) by *N*-methyl-phenylalanine (MePhe), a structural unit of the *in-vivo* active beauvericin, significantly improved its anthelmintic activity *(27)*. The resulting enniatin **27b** (Figure 12) tested *in vivo* was found to be fully active against the gastrointestinal nematode *Haemonchus contortus* in sheep at an oral dosage of 5.0 mg/kg body weight.

Cyclo(MeIle-*(R)*-Hylv-MeIle-*(R)*-Hylv-**MeXaa**-*(R)*-Hylv-MeIle)

27a: MeXaa = MeIle, **27b:** MeXaa = MePhe

Figure 12. Synthetic enniatin A derivative 27b

Surprisingly, on the basis of an indication shift (e.g., from anthelmintic to fungicidal activity) the novel enniatin **27b** also shows much better fungicidal activity against the ascomycete *B. cinerea*.

b) Amblyomycin: Parasitism between different fungi is called mycoparasitism. If the host is also a parasitic organism, hyperparasitism exists, as in the outlined example. Dual culture of the phytopathogenous fungus host *B. cinerea* and the

Downloaded by UNIV OF GUELPH LIBRARY on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch010

mycoparasite *Amblyosporium spongiosum* leads to a novel fungitoxic metabolite, the cyclic nonapeptide amblyomycin **28**. It contains the *(R)*-2-aminodecanic acid (*R*-Dec) which is so far unknown in naturally occurring peptides. The nonapeptide **28** shows fungicidal activity against *Sphaerotheca fuliginea* in cucumber, *Venturia inaequalis* in apple as well as *Erysiphe graminis* in barley, *Leptinospaera nodorum* in wheat, and *B. cinerea* in pepper and beans (Figure 13).

Cyclo(Ala-Asn-*(R)*-Asn-*(R)*-Leu-Thr-*(R)* -Leu-***(R)***-**Dec**-Orn-Ala)

*Figure 13. Amblyomycin **28***

Unfortunately, numerous NPs described above have insufficient activity in field screening.

Modern and Complementary Technologies

NP research is an interdisciplinary effort. Therefore, the starting point for the identification of new biologically active NPs is the large *in-house* strain collection held in Bayer HealthCare.

After cultivation of the biological material, extraction of a crude component mixture and enrichment of selected ingredients using modern purification techniques, automated sample provision of separated ingredients and transfer to well-plate format for robotic high throughput screening is carried out.

Today, *in-vivo* and *in-vitro* Ultra High Throughput Screening (UHTS) bioassays with capacities of >100,000 compounds per day have been installed and a target based approach using mechanism-based screens has replaced non-specific assays. Receptor- or enzyme-based screens with a target of special interest for crop protection are now used. Thus, *in-vivo* and *in-vitro* hits can be selected from the test libraries, and are subsequently validated in microscreening assays in order to identify a real *in-vivo* hit. These steps are followed by profiling of the candidate in greenhouse and field trials, and identification of a lead structure, which will be further optimized in research projects and later on in development projects. The driving force is reducing the time to market for new a.i.s. This requires dramatic performance improvements in lead discovery and further development of selected candidates.

Concluding Remarks

Despite of the low number of NPs currently on the market, their agricultural market share is remarkable. Bayer CropScience has long experience in the design of biologically active NPs for the current main agrochemical areas; there

Downloaded by UNIV OF GUELPH LIBRARY on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch010

are several products on the market. The molecular diversity of NPs isolated from various species is a dominant factor in the discovery process for novel structures and has offered completely new approaches for lead-finding as well. In this connection the improvement of the dereplication process (the rapid identification and elimination of known compounds or previously identified chemical isolates present in crude natural product extracts), novel and complementary technologies and screening methods for a high number of compounds are powerful tools in the search for unknown a.i.s in modern crop protection.

During evolution, life forms on earth have synthesized a wide variety of chemical compounds to protect themselves from being eaten, or in order to adapt to environmental conditions. Around 2 million life forms have been discovered so far, yet the real number is bound to be much higher. The most contentious point in the scientific community is the number of animal species - particular insects. Some estimates put the number between 3 and 6 million, other put it as higher as 30 million. Generally, undiscovered NPs can be a great chance for crop protection to identify novel a.i.s. Therefore, prospects of NP chemistry will focus:

- Exploration of this rich source for high structural diversity.
- Discovery of innovative molecular targets and leads for agrochemicals.
- Rationally driven search for bioactive ingredients with new MoA.

Only a few compounds have been investigated up to now - this could be the challenge for the future of the agrochemical industry.

Downloaded by UNIV OF GUELPH LIBRARY on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch010

Acknowledgements

The material covered by this article is based on the inventions and scientific support of many colleagues in Bayer CropScience AG.

References

1. Tomlin, C. D. S. (ed), *The Pesticide Manual*, 12th edition, British Crop Protection Council, Bracknell **2000**.
2. Staudinger, H.; Ruzicka, L. *Helv. Chim. Acta* **1924**, *7*, 177-201.
3. Narahashi, T. *Residue Rev.* **1969**, *25*, 275-288.
4. Elliott, M.; Farnham, A. W.; Janes, N. F.; Needham, D. H.; Pulman, D. A. *Nature* **1974**, *248*, 710-711.
5. Naumann, K. In: *Chemistry of Plant Protection 4*, *Synthetic Pyrethroid Insecticides* (Eds: W. S. Bowers, W. Ebing, D. Martin, R. Wegeler), Springer, **1990**, pp. 43.
6. Breese, M. H.; Highwood, D. P. *BCPC Conf. Pest Dis.* **1977**, *2*, 641-648.

7. Pinochet, G. *Phytoma* **1991**, *428*, 54-57.
8. Anke, T.; Schramm, B.; Schwalge, B.; Steffan, B.; Steglich, W. *Liebigs Ann. Chem.* **1984**, 1616-1625.
9. Anke, T.; Besl, H.; Mocek, U.; Steglich, W. *J. Antibiotics* **1983**, *36*, 661-666.
10. Sauter, H.; Steglich, W.; Anke, T. *Angew. Chem., Int. Ed.* **1999**, *38*, 1329-1349.
11. Becker, W. F., von Jagow, G.; Anke, T.; Steglich, W. *FEBS Lett.* **1981**, *132*, 329-333.
12. Frank, J. A.; Sanders, P. L. *BCPC Conf. Pests Dis.* **1994**, *2*, 871-876.
13. Margot, P.; Huggenberger, F.; Amrein, J.; Weiss, B. *BCPC Conf. Pests Dis.* **1998**, *2*, 375-382.
14. Dutzmann, S.; Mauler-Machnik, A.; Kerz-Möhlendick, F.; Applegate, J.; Heinemann, U. *BCPC Conf. Pests Dis.* **2002**, *1*, 365.
15. Fugmann, B.; Lieb, F.; Moeschler, H.; Naumann, K.; Wachendorf, U. *Chemie in Unserer Zeit* **1992**, *26*, 35-41.
16. Velten, R.; Erdelen, C.; Gehling, M.; Göhrt, A.; Gondol, D.; Lenz, J.; Lockhoff, O.; Wachendorff, U.; Wendisch, D. *Tetrahedron Lett.* **1998**, *39*, 1737-1740.
17. King, M. L.; Chiang, C. C.; Ling, H. C.; Fujita, E.; Ochiai, M.; McPhail, A. T. *J. Chem. Commun.* **1982**, 1150-1151.
18. Schoop, A.; Greiving, H.; Göhrt, A. *Tetrahedron Lett.* **2000**, *41*, 1913-1916.
19. Mayer, A.; Kilian, M.; Hoster, B.; Sterner, O.; Anke, H. *Pestic. Sci.* **1999**, *55*, 27-30.
20. Thern, B.; Rudolph, J.; Jung, G. *Angew. Chem.* **2002**, *114*, 2401-2403.
21. Falb, E.; Yechezkel, Y.; Salitra, Y.; Gilon, C. *J. Pept. Res.* **1999**, *53*, 507-517.
22. Sewald, N. *Angew. Chem.* **2002**, *114*, 4855-4857.
23. Mühlbauer, A.; Seip, S.; Nowak, A.; Tran, V. S. *Helv. Chim. Acta* **2003**, *86*, 2065-2072.
24. Burton, J. D. *Herbic. Act.: Toxicol. Biochem. Mol. Biol.* **1997**, 187-205.
25. Jeschke, P.; Etzel, W.; Harder, A.; Schindler, M.; Göhrt, A.; Pleiss, U.; Kleinkauf, H.; Zocher, R.; Thielking, G.; Gau, W.; Bonse, G., In: *Bioorg. Chem.* **1999**, 207-214. Editor(s): Diederichsen, Ulf. Wiley-VCH Verlag GmbH, Weinheim, Germany.
26. Pleiss, U.; Turberg, A.; Harder, A.; Londershausen, M.; Jeschke, P.; Boheim, G. *J. Labelled Compd. Radiopharm.* **1996**, *38*, 651-659.
27. Scherkenbeck, J.; Jeschke, P.; Harder, A. *Current Topics in Med. Chem.* (Hilversum, Netherlands) **2002**, *2*, 759-777.

Downloaded by UNIV OF GUELPH LIBRARY on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch010

Downloaded by UNIV OF GUELPH LIBRARY on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch011

Chapter 11

Phytotoxic, Antialgal, and Antifungal Activity of Constituents from Selected Plants of Kazakhstan

Mozaina Kobaisy[1], Maria R. Tellez[1], Kevin K. Schrader[1], David E. Wedge[1], Gulnara T. Sitpaeva[1,4], Nadejda G. Gemejieva[2], Gaukhar S. Mukanova[3], and Leonid K. Mamonov[3]

[1]Natural Products Utilization Research Unit, Agricultural Research Service, U.S. Department of Agriculture, P.O. Box 8048, University of Mississippi, Oxford, MS 38677–8048
[2]Institute of Botany and Phytointroduction, Almaty, Kazakhstan
[3]Institute of Plant Physiology, Almaty, Kazakhstan
[4]Current address: Inter Science Institute, 944 West Hyde Park Boulevard, Inglewood, CA 90302

Ninety two extracts representing 23 species of plants endemic to Kazakhstan have been investigated for their phytotoxic, antialgal, and antifungal activities. Twenty extracts were found to have antifungal activity against one or more of the pathogenic fungi *Colletotrichum accutatum*, *C. fragariae*, and *C. gloeosporioides*. Two extracts of *Verbascum songoricum* demonstrated good activity against the cyanobacterium *Oscillatoria perornata*. Bioactivity-guided fractionation of the water extract of *V. songaricum* afforded aucubin as the active algaecidal compound. Extracts prepared from *Artemisia terrae-albae*, *Geranium collinum*, *Haplophyllum sieversii*, and *Tamarix arceuthoides* were phytotoxic to bentgrass, but not to lettuce seedlings. Analysis of the extract from of *Nepeta pannonica* obtained by supercritical fluid extraction resulted in the identification of the major components 1,8-cineole and *cis,cis*-nepetalactone.

In Natural Products for Pest Management; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

Plants produce chemicals to protect themselves against microbial pathogens, nematodes, a wide range of herbivores, and other plant species that compete for resources. Due to the prohibitive cost of synthetic agrochemicals and the problems of environmental pollution caused by continuous use of these chemicals, there is a renewed interest in the use of botanicals in agriculture (*1*,*2*). A number of agricultural chemists, entomologists, nematologists and pathologists all over the world are now actively engaged in research into usage of plant products against agricultural pests and diseases to minimize losses caused by them. Systematic screening of plants from Kazakhstan for antifungal, antialgal, as well as for phytotoxic activity resulted in the discovery of active extracts and compounds.

Downloaded by UNIV OF GUELPH LIBRARY on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch011

Materials and Methods

Plant collection. Plants were collected in Kazakhstan (Table I), from the east part of mountain ridge Malaysary passes Tasmurun and Koulanbasy. In order to ensure accurate botanical identifications, only plants that were in flower were collected.

Preparation of extracts. Air dried plant samples were cut into pieces, crushed in using a grinding mill and sequentially extracted three times with pentane, pentane:acetone:water (54:44:2), ethanol, and water. Solvents were removed under vacuum except for water that was removed by freeze-drying.

Carbon dioxide extractions were conducted with a supercritical fluid extractor (SFE) (Applied Separations, Allentown, PA). About 18-20 g of powdered dried plant material was added to a 50 mL stainless steel extraction cell with glass fiber filter disks (18 mm diameter) on the top and bottom of the cell. Experiments were run at a pressure of 6000 psi and a temperature of 100 °C. There was a 10 min static hold followed by a dynamic extraction at a rate of ca. 2.5 L expanded CO_2 per minute. The variable restrictor was heated to 70°C, and extracts were collected every 10 L of CO_2 up to 80 L in 12 mL glass vials. SFE/supercritical fluid chromatography (SFC) grade CO_2 was used for all extractions.

Isolation of the active constituents was done using bioassay-guided procedures. Various combinations of chromatographic methods and solvent systems were used to separate active constituents. Compounds structures were elucidated by a combination of NMR and MS spectrometric techniques.

Analysis of the SFE-CO_2 extract of *Nepeta pannonica* was performed by GC-MS on a Varian CP-3800 gas chromatograph coupled with a Varian Saturn 2000 GC/MS. Details of the analyses will be reported elsewhere.

Table I. Plants Collected in Kazakhstan.

Species	Family	Part
Achillea millefolium	Asteraceae	aerial part
Aconitum leucostomum	Ranunculaceae	root
Artemisia leucodes	Asteraceae	aerial part
Artemisia terrae-albae	Asteraceae	aerial part
Clematis orientalis	Ranunculaceae	aerial part
Clematis songarica	Ranunculaceae	aerial part
Delphinium semibarbatum	Ranunculaceae	aerial part
Eremurus inderiensis	Liliaceae	root
Eremurus fuscus	Liliaceae	aerial part
Euphorbia latifolia	Euphorbiaceae	root
Ferula ferulaeoides	Apiaceae	aerial part
Ferula leiophylla	Apiaceae	aerial part
Ferula stylosa	Apiaceae	aerial part
Geranium collinum	Geraniaceae	aerial part
Haplophyllum sieversii	Rutaceae	aerial part
Iris ruthenica	Iridaceae	aerial part
Iris songorica	Iridaceae	root
Juniperus pseudosabina	Cupressaceae	aerial part
Linaria vulgaris	Scrophulariaceae	aerial part
Nepeta pannonica	Lamiaceae	aerial part
Tamarix arceuthoides	Tamaricaceae	aerial part
Verbascum blattaria	Scrophulariaceae	aerial part
Verbascum songaricum	Scrophulariaceae	root

Bioassays

Three different bioassays were performed for each plant extract.

1- Algicidal assays. A rapid bioassay (*3*) was used to determine the Lowest-Observed-Effect Concentration (LOEC) and the Lowest-Complete-Inhibition Concentration (LCIC) of the plant extracts and components towards isolates of the cyanobacterium (blue-green alga) *Oscillatoria perornata* Sluja, and the green alga *Selenastrum capricornutum* Printz. A 96-well quartz microplate (Hellma Cells, Inc., Forest Hills, New York) was used for all SFE-CO2 extracts, since hexane was the loading solvent and is incompatible with polystyrene microplates (*4*).

2- Phytotoxicity assays. Bioassays for phytotoxic activity were carried out as previously reported for lettuce (*Lactuca sativa* cv. Iceberg) and bentgrass (*Agrostis stolonifera* cv. Pencross) in 24-well plates (*5*), except that loading solvents were allowed to evaporate completely at room temperature (5 min)

Downloaded by UNIV OF GUELPH LIBRARY on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch011

before dilution with water (*6*). An added control of the loading solvent (evaporated at room temperature) was used to account for possible solvent effects. The phytotoxicity was rated on a scale ranging from 0 to 5. Ratings of 0 and 5 meant no effect and complete inhibition, respectively.

3- Fungicidal assays. Pathogen production and inoculum preparation for *Colletotrichum fragariae* Brooks, *Colletotrichum gloeosporioides* Penz. & Sacc., and *Colletotrichum acutatum* Simmonds were carried out as described elsewhere (*7*). Conidia concentrations were determined photometrically (*7,8*) from a standard curve and suspensions were then adjusted with sterile distilled water to a concentration of 1.0 x 10^6 conidia/mL. Bioautographic assays (*9*) were chosen to determine the inhibition of fungal growth on thin layer chromatography (TLC) plates. Extracts were spotted on a TLC plate using a disposable glass micropipette. In order to evaluate the majority of the antifungal constituents, 400.0 µg of each extract was used as the loading amount during the prescreening phase. Subsequent secondary evaluations of positive antifungal extracts will be conducted at appropriate concentrations for agrochemical applications. Loading solvents were used as controls. The experiment was repeated 3 times. To detect biological activity directly on the TLC plate, the silica gel plates (250 µ, Silica Gel GF Uniplate, Analtech, Inc., Newark, DE) were sprayed with a spore suspension as previously described (*10*). Positive antifungal activities were established by the presence of measurable zones of growth inhibition. The antifungal activity was recorded as the diameter of the clear zone (in millimeters) after 4 days of incubation for each fungus.

Downloaded by UNIV OF GUELPH LIBRARY on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch011

Results and Discussion

Algicidal Activity

Extracts were screened against one cyanobacterium (blue-green algae) and one green alga for potential as a selective cyanobactericide. Off-flavor in channel catfish [*Ictalurus punctatus* (Rafinesque)] raised in the southeastern United States creates an unpalatable and, therefore, unmarketable product that results in large economic losses to the industry. Most off-flavor episodes in catfish are attributed to their absorption of earthy/musty compounds produced by certain species of cyanobacteria that grow in the catfish production ponds. In west Mississippi, the cyanobacterium *Oscillatoria perornata* (Skuja), a producer of the musty-odor compound 2-methylisoborneol (MIB), is attributed with being the major cause of musty off-flavor in farm-raised catfish (*11*). Green algae are not associated with such undesirable metabolites and are also preferable to cyanobacteria in catfish production ponds because they are better oxygenators of the water and a better base for aquatic food chains (*12*). Therefore, the discovery of safe compounds that selectively kill cyanobacteria would greatly benefit the channel catfish industry.

All of the extracts from plant species listed in Table I had no activity against both *O. perornata* and *S. capricornutum*, having shown LOEC and LCIC values >100, except for two extracts from *Verbascum songaricum* (SFE-CO_2 and H_2O), which showed good activity towards *Oscillatoria perornata* with LCIC of 10 ppm.

The genus *Verbascum* (Scrophulariaceae) is widespread throughout the Mediterranean region. It comprises over 270 species, some of which are widely used as European folk medicines (*13*). *Verbascum songaricum*, a perennial plant growing wild in many parts of Central Asia, is also among traditional medicines in Russia (*14*).

Bioactivity-guided fractionation of *V. songaricum* H_2O extract afforded aucubin (**1**) as the major active compound, but is not selectively inhibitory towards blue-green algae (Table II). The separation of the iridoid glycosides **1** and harpagoside (**2**), was achieved by column chromatography on silica gel and HPLC on RP-18 column. The phenylpropanoid glycosides verbascoside (**3**), and ligupurpuroside A (**4**) (Figure 1) were also isolated from the active fraction but did not have good antialgal properties.

Table II. LOEC of Compounds Screened against *O. perornata* and *S. capricornutum*.

Compound	LOEC (μM) *O. perornata*	LOEC (μM) *S. capricorco*
Aucubin	1.0	1.0
Harpagoside	100	100
Verbascoside	100	>100
Ligupurpuroside A	100	>100

Phytotoxic Activity

Fourteen extracts from eight plant species had phytotoxic activity (Table III). Six of these extracts (obtained from *Artemisia terrae-albae*, *Geranium collinum*, *Haplophyllum sieversii*, and *Tamarix arceuthoides*) were phytotoxic to bentgrass but not to lettuce.

Nepeta pannonica was of interest because its SFE-CO_2 extract showed strong phytotoxic activity to both bentgrass and lettuce seedlings. Furthermore, many species in this genus (Lamiaceae), comprising about 280 species, have reported biological activities, and are likewise used in folk medicine as bacteriostatic and disinfectants, as well as against eczema-type skin disorders (*15*).

GC/MS analysis of the SFE-CO_2 extract of *Nepeta pannonica* revealed the presence of 1,8-cineole (**5**), and 4a-α,7-β,7a-α-nepetalactone (**6**) as the major

Downloaded by UNIV OF GUELPH LIBRARY on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch011

Downloaded by UNIV OF GUELPH LIBRARY on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch011

constituents (Figure 1); However, **6** , which was obtained by column chromatogtaphy on silica gel but did not display phytotoxic activity even at a treatment level as high as 2 mg/mL. The phytotoxicity of the SFE-CO_2 extract is probably due to the presence of the known phytotoxin 1,8-cineole (*16-18*), which is present as the other major component in the oil.

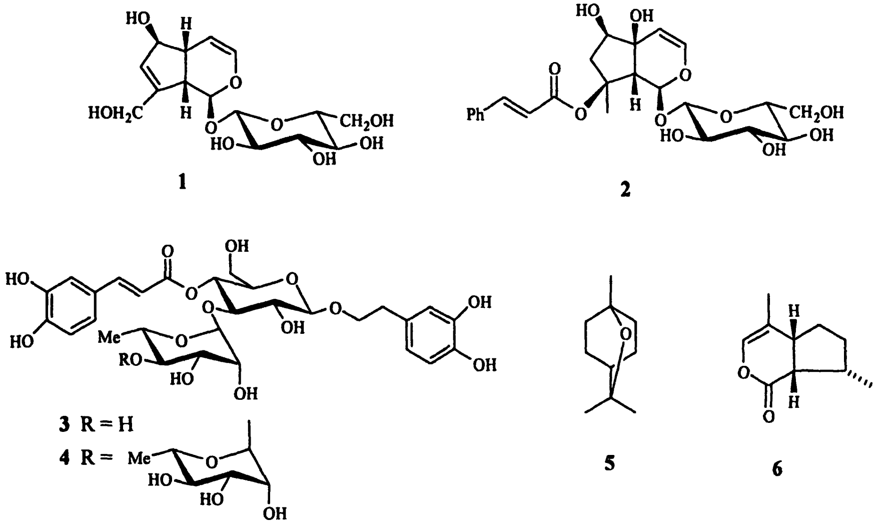


Figure 1. Structures of compounds isolated.

Fungicidal Activity

Filamentous fungi of the genus *Colletotrichum* and its teleomorph *Glomerella* are major plant pathogens worldwide. *Colletotrichum* species often cause typical symptoms of anthracnose, a disease characterized by sunken necrotic lesions usually bounded by a red margin (*19*, *20*). The pathogens *Colletotrichum acutatum* J. H. Simmonds, *C. gloeosporioides* Penz., and *C. fragariae* A. N. Brooks can occur singly or in combination and can infect flowers, fruit, leaves, petioles, stolons, and crowns (*21*, *22*).

Twenty extracts representing 10 plant species were found to have antifungal activity against one or more of the pathogenic fungi *Colletotrichum accutatum*, *C. fragariae*, and *C. gloeosporioides* (Table IV). Several of these extracts are now under investigation.

Table III. Activity of Phytotoxic Plants.

Species	Extracts	Visual rating[a] Lettuce	Visual rating[a] Bentgrass
Achillea millefolium	SFE-CO_2	0	0
	PAW54:44:2	4	5
	Ethanol	0	3
	H_2O	0	3
Artemisia leucodes	SFE-CO_2	0	0
	PAW54:44:2	3	3
	Ethanol	0	0
	H_2O	0	0
Artemisia terrae-albae	SFE-CO_2	0	0
	PAW54:44:2	0	4
	Ethanol	0	0
	H_2O	0	0
Eremurus inderiensis	SFE-CO_2	3	4
	PAW54:44:2	5	4
	Ethanol	0	0
	H_2O	0	0
Geranium collinum	SFE-CO_2	0	3
	PAW54:44:2	0	3
	Ethanol	0	0
	H_2O	0	0
Haplophyllum sieversii	SFE-CO_2	0	3
	PAW54:44:2	0	3
	Ethanol	0	0
	H_2O	0	0
Juniperus pseudosabina	SFE-CO_2	3	3
	PAW54:44:2	0	0
	Ethanol	0	0
	H_2O	0	0
Nepeta pannonica.	SFE-CO_2	5	4
	PAW54:44:2	0	0
	Ethanol	0	0
	H_2O	0	0
Tamarix arceuthoides	SFE-CO_2	0	0
	PAW54:44:2	0	3
	Ethanol	0	0
	H_2O	0	0

[a] Visual rating scale ranges from 0 to 5 for no effect to 100% inhibition, treatment 1 mg/mL.

Downloaded by UNIV OF GUELPH LIBRARY on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch011

Downloaded by UNIV OF GUELPH LIBRARY on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch011

Table IV. Fungal Growth Inhibition of the Active Plants on *Colletotrichum gloeosporioides*, *C. fragaria*, and *C. acutatum*.

		Diameter of zone of inhibition in mm[a]		
	Extracts	*C. gloeoporioides*	*C. fragariae*	*C. acutatum*
Aconitum leucostomum	SFE-CO_2	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)
	PAW54:44:2	11.0 (±1.0)	13.0 (±0.5)	12.0 (±0.7)
	Ethanol	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)
	H_2O	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)
Delphinium semibarbatum	SFE-CO_2	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)
	PAW54:44:2	5.0 (±0.6)	7.0 (±1.0)	5.0 (±0.7)
	Ethanol	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)
	H_2O	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)
Eremurus inderiensis	SFE-CO_2	9.0 (±0.6)	9.0 (±0.7)	8.0 (±0.7)
	PAW54:44:2	10.0 (±1.0)	10 .0 (±0.7)	8.0 (±0.9)
	Ethanol	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)
	H_2O	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)
Eremurus fuscus	SFE-CO_2	0.0 (±0.0)	7.0 (±0.7)	0.0 (±0.0)
	PAW54:44:2	0.0 (±0.0)	11.0 (±1.0)	0.0 (±0.0)
	Ethanol	0.0 (±0.0)	13.0 (±0.8)	0.0 (±0.0)
	H_2O	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)
Ferula leiophylla	SFE-CO_2	10.0 (±0.7)	8.0 (±0.8)	11.0 (±0.8)
	PAW54:44:2	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)
	Ethanol	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)
	H_2O	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)
Geranium collinum	SFE-CO_2	0.0 (±0.0)	0.0 (±0.0)	5.0 (±0.9)
	PAW54:44:2	0.0 (±0.0)	5.0 (±0.9)	8.0 (±0.9)
	Ethanol	0.0 (±0.0)	0.0 (±0.0)	5.0 (±0.8)
	H_2O	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)
Haplophyllum sieversii	SFE-CO_2	9.0 (±0.7)	9.0 (±0.7)	13.0 (±0.6)
	PAW54:44:2	10.0 (±0.5)	10.0 (±0.9)	7.0 (±0.5)
	Ethanol	9.0 (±0.1)	11.0 (±0.5)	7.0 (±0.9)
	H_2O	9.0 (±1.0)	7.0 (±0.7)	11.0 (±0.7)
Iris songorica	SFE-CO_2	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)
	PAW54:44:2	8.0 (±1.0)	8.0 (±0.9)	10.0 (±0.9)
	Ethanol	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)
	H_2O	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)
Juniperus pseudosabina	SFE-CO_2	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)
	PAW54:44:2	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)
	Ethanol	5.0 (±0.7)	6.0 (±0.5)	8.0 (±0.8)
	H_2O	7.0 (±0.9)	9.0 (±0.9)	11.0 (±0.8)
Verbascum songaricum	SFE-CO_2	8.0 (±0.7)	7.0 (±1.0)	8.0 (±0.9)
	PAW54:44:2	8.0 (±1.0)	10.0 (±0.7)	9.0 (±0.9)
	Ethanol	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)
	H_2O	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)

[a]Mean zone of fungal growth inhibition. Standard deviation is within parenthesis.

Conclusion

Our results demonstrate the potential of Central Asian plants as sources of pesticides. Although phytochemical investigations of the plants we have selected may have been conducted previously, most of the pesticidal properties of these plants are described here for the first time. The aqueous, organic, and SFE-CO_2 extracts have, in general, shown similar activities; thus it is not feasible to conclude that any one method of extraction is preferable.

Downloaded by UNIV OF GUELPH LIBRARY on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch011

Acknowledgements

This research was funded in part by the ARS for the Former Soviet Union Program, K-790p. We would like to thank Amber Callahan, Ramona Pace, and Linda Robertson for their valuable technical support.

References

1. Pillmoor, J. B.; Wright, K.; Terry, A. S. *Pestic. Sci.* **1993**, *39*, 131-140.
2. Isman, M. B. *Rev. Pestic. Toxicol.* **1995**, *3*, 1-20.
3. Schrader, K. K.; de Regt, M. Q.; Tucker, C. S.; Duke, S. O. *Weed Technol.* **1997**, *11*, 767-774.
4. Tellez, M. R.; Khan, I. A.; Kobaisy, M.; Schrader, K. K.; Dayan, F. E; Osbrink W. *Phytochemistry* **2002**, *61*, 149-55.
5. Dayan, F. E.; Romagni, J. G.; Duke S. O. *J. Chem. Ecol.* **2000**, *26*, 2079-2094.
6. Kobaisy, M.; Tellez, M. R.; Dayan, F. E.; Duke, S. O. *Phytochemistry* **2002**, *61*, 37-40.
7. Wedge D. E.; Kuhajek, J. M. *SAAS Bulletin Biochem. Biotech.* **1998**, *11*, 1-7.
8. Espinel-Ingroff A.; Kerkering T. M. *J. Clin. Microbiol.* **1991**, *29*, 393-394.
9. Homans A. L.; Fuchs A. *J. Chromatogr.* **1970**, *51*, 327-329.
10. Wedge, D. E.; Nagle, D. G. *J. Nat. Prod.* **2000**, *63*, 1050-1054.
11. van der Ploeg, M.; Dennis, M. E.; and de Regt, M. Q. *Water Sci. Technol.* **1995**, *31*, 173-180.
12. Paerl, H. W.; Tucker, C. S. *J. World Aquacult. Soc.* **1995**, *26*, 109-131.
13. Bianco, A.; Guiso, M.; Passacantilli, P. *J. Nat. Prod.* **1984**, *47*, 901-902.
14. Hartleb, I.; Seifert, K. *Phytochemistry* **1995**, *38*, 221-224.

15. De Pooter, H. L.; Nicolai, B.; De Buyck, L. F.; Goetghebeur, P.; Schamp, N. M. *Phytochemistry* **1987**, *26*, 2311-2314.
16. Romagni, G. J.; Allen, S. N.; Dayan, F. E. *J. Chem. Ecol.* **2000**, *26*, 303-313
17. Romagni, J. G.; Duke, S. O.; Dayan, F. E. *Plant Physiol.*, **2000**, *123*, 725-732.
18. Singh, H. P.; Batish, D. R.; Kohli, R. K. *Crop Protection* **2002**, *21*, 347-350.
19. Freeman, S.; Katan, T.; and Shabi, E. *Plant Dis.* **1998**, *82*, 596-607.
20. Wedge, D. E.; Riley, M. B.; and Tainter, F. H. *Plant Dis.* **1999**, *83*, 377-380.
21. Howard, C. M.; Maas, J. L.; Chandler, C. L. *Plant Dis.* **1992**, *76*, 976-981.
22. Smith, B. J. In *Compendium of Strawberry Diseases*; J. L. Maas, Ed.; American Phytopathological Society: St. Paul, MN, 1998; pp 24-48.

Downloaded by UNIV OF GUELPH LIBRARY on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch011

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch012

Chapter 12

Finding New Fungicides from Natural Sources

David E. Wedge and Stephen O. Duke

Natural Products Utilization Research Unit, Agricultural Research Service, U.S. Department of Agriculture, P.O. Box 8048, University, MS 38677–8048

Discovery of natural product fungicides is largely dependent upon the availability of high quality miniaturized antifungal bioassays. As part of a program to discover natural product-based fungicides, several sensitive assay systems were developed for evaluation of naturally occurring antifungal agents. Bioautography was used to identify the number of antifungal compounds in plant extracts, eliminate nuisance compounds, and characterize antifungal activity of semipure compounds. Bioautography techniques were successful in evaluating naturally occurring quinones and antifungal compounds from strawberry, *Artemisia douglasiana,* and *Pimpinella* species. A second bioassay using a 96-well microtiter format was developed to evaluate partially purified active fractions and pure compounds identified by bioautography. The 96-well plate assay has allowed us to study antifungal activity of sesquiterpene lactones, CAY-1 (steroidal saponin), sampangine (alkaloid), and compounds from *Ruta graveolens*. This coupled approach to natural product discovery combines the simple and visual nature of direct bioautography with the rapid, sensitive, and high-throughput capabilities of a microtiter system. This review covers bioautography prescreens, microtiter assays (secondary assays), and detached leaf assays for natural product effects on strawberry anthracnose (*Colletotrichum* spp.).

U.S. government work. Published 2006 American Chemical Society

In Natural Products for Pest Management; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

Since the early 1970s, U.S. and worldwide agriculture have struggled with the evolution of pathogen resistance to antimicrobial disease control agents. Increased necessity for repeated chemical applications, development of pesticide cross-resistance, and disease resistance management strategies has overshadowed the use of agricultural chemicals. Producers are currently attempting to control agricultural pests with fewer effective chemicals. In addition, the desire for pesticides with greater environmental toxicological safety is a major public concern. Particularly desirable is the discovery of novel pesticidal agents representing new chemical classes that operate by different modes of action and, consequently, target organisms with resistance to currently used chemicals. In this respect, evaluating natural products and extracts to identify potential new pesticides offers an approach to discover new chemical entities that have never been synthesized by chemists or evaluated by biologists.

Antibiotics, antineoplastics, herbicides, and insecticides often originate from plant and microbial chemical defense mechanisms (*1*). Secondary metabolites, once considered unimportant products, are now thought to mediate chemical defense mechanisms by providing chemical barriers against animal and microbial predators (*1, 2*). Plants produce numerous chemicals for defense and communication, but plants can also generate their own form of offensive chemical warfare targeting proliferation of pathogens. These chemicals may have general or specific activity against key target sites in bacteria, fungi, and viruses. The chemical warfare between plants and their pathogens shows promise to provide natural products as new anti-infectives for human and animal health, as well as for agricultural pest control. The recent successful development of strobilurin fungicides and spinosad insecticides has spurred the interest in natural products as crop protectants. The importance and future of natural product fungicides is seen by the fact that 21 companies have filed 255 patent applications primarily for use as strobilurin class fungicides (Qo I MET complex 3 inhibitors).

Plants, fungi, and other marine and terrestrial organisms produce hundreds of thousands of compounds that often suppress invasion and growth of pathogenic microorganisms. Our goal is to rapidly identify compounds that are active at low rates (≤ 3 μM) and will have the greatest commercial potential as agrochemicals or pharmaceuticals.

Compounds that Act Directly on Pathogens

Since the discovery of the vinca alkaloids in 1963, many of the major known antitubulin agents used in today's cancer chemotherapy arsenal are products of plant secondary metabolism. These compounds probably act as defense

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch012

chemicals that target and inhibit cell division in invading pathogens (*1*). Therefore, it is reasonable to hypothesize that plants produce chemicals such as strobilurin and resveratrol that act in defense directly by inhibiting pathogen proliferation, or indirectly by disrupting chemical signal processes related to growth and development of pathogens or herbivores (*1*).

Compounds that Induce Resistance Mechanisms in Plants

Plant resistance to pathogens is considered to be systemically induced by some endogenous signal molecule produced at the infection site that is then translocated to other parts of the plant (*3*). Discovery of the putative signal is of great interest to many plant scientists because such molecules have possible uses as "natural product" disease control agents. However, research indicates that there is not a single compound but a complex signal transduction pathway in plants that can be mediated by a number of compounds that appears to influence octadecanoid metabolism. In response to wounding or pathogen attack, fatty acids of the jasmonate cascade are formed from membrane-bound α-linolenic acid by lipoxygenase- mediated peroxidation (*4*). Analogous to the prostaglandin cascade in mammals, α-linolenic acid is thought to participate in a lipid-based signaling system where jasmonates induce the synthesis of a family of wound-inducible defensive proteinase inhibitors (*5*) and low and high molecular weight phytoalexins such as flavonoids, alkaloids, and terpenoids (*6,7*).

Several natural products from plants and bacteria have applications as plant protectants through induction of systemic acquired resistance (SAR), a process which includes production of natural antimicrobials (phytoalexins) by the plant. In particular, cell wall components of fungi and some algae induce SAR. For example, chitin (either from fungi or arthropods) was one of the first commercial elicitors of SAR (*8*, 9). Some polysaccharides are also good SAR inducers (*10*, *11*), but these have not been marketed to our knowledge.

Other commercial products that induce SAR include Messenger® (EDEN Biosciences, Inc., Bothell, WA) and Milsana® (KHH BioSci, Inc., Raleigh, NC). Messenger® is a harpin protein which switches on natural plant defenses in plant response to bacterial leaf spot, wilt, and blight and fungal diseases such as botrytis, brunch rot, and powdery mildew (*12*). Milsana® is a crude extract from *Reynoutria sachalinensis* (giant knotweed) which induces phytoalexins that confer resistance to powdery mildew and other diseases such as *Botrytis* spp. (*13*). The active component of this extract may be formed during extraction by acid hydrolysis of a natural compound (*14*). However, some of the compounds in this extract, such as emodin (*15*), are known to have direct antimicrobial activity (*16*).

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch012

Materials and Methods

Biological Evaluation of Antifungal Activity

Many experimental approaches have been used to screen compounds and extracts from plants and microorganisms in order to discover new antifungal compounds. The focus of this chapter is the bioassay methods that we are currently using in our laboratories to evaluate antifungal compounds produced by plants, their pathogens, and other terrestrial and marine organisms. As part of a program to discover and develop natural product-based fungicides, new sensitive detection systems were developed for direct and *in planta* evaluations of antifungal agents. However, elicitors with no innate antifungal activity will not appear active in most current high-throughput screening systems.

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch012

Pathogen Production

Conidia are harvested from 7-10 day-old cultures by flooding plates with 5 mL of sterile distilled water and softly brushing the colonies with an L-shaped glass rod. Aqueous conidial suspensions are filtered through sterile Miracloth (Calbiochem-Novabiochem Corp., La Jolla CA) to remove mycelia. Conidia concentrations are determined photometrically (*17, 18*) from a standard curve based on the absorbance at 625 nm, and suspensions are then adjusted with sterile distilled water to a concentration of 1.0 x 10^6 conidia/mL.

Standard conidial concentrations are determined from a standard curve for each fungal species. Standard turbidity curves are periodically validated using both a Bechman/Coulter Z1 (Fullerton, CA) particle counter and hemocytometer counts. Conidial and mycelial growth are evaluated using a Packard Spectra Count (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA). Conidial growth and germ tube development are evaluated using an Olympus IX 70 (Olympus Industrial America, Inc., Melville, New York) inverted microscope and recorded with a Olympus DP12 digital camera as appropriate for compounds that affect spore germination and early germ tube development.

Direct Bioautography

Bioautography techniques are used as primary screening systems to detect antifungals. Matrix, one-dimensional, and two-dimensional bioautography protocols on silica gel thin layer chromatography (TLC) plates with *Colletotrichum spp.* used as the test organisms were used to identify the antifungal activity according to the published methods (*19-21*). Matrix bioautography is used to screen large numbers of crude extract at 20 mg/mL.

One-dimensional TLC (1D- TLC) and two-dimensional TLC (2D-TLC) are subsequently used to separate and identify the number of antifungal agents in an extract.

A 2D-TLC direct bioautography method is used to evaluate active crude and partially purified chromatographic fractions. This protocol utilizes two sequential TLC runs in which the TLC plates are developed once with a polar solvent, turned 90°, and then developed a second time with a non-polar solvent system (*21*). The method takes advantage of the resolving power of 2D-TLC to separate chemically diverse mixtures found in crude extracts. Two-dimensional TLC bioautography is well suited to resolving extracts containing lipophilic natural products that are difficult to separate by single elution TLC.

Each plate is subsequently sprayed with a spore suspension (10^5 spores/mL) of the fungus of interest and incubated in a moisture chamber for three days at 26 °C with a 12 h photoperiod. Clear zones of fungal growth inhibition on the TLC plate indicate presence of antifungal constituents in each extract. Inhibition of fungal growth is evaluated 3-4 days after treatment. Antifungal metabolites can be readily located on the plates by visually observing clear zones where the active compounds inhibit fungal growth (*21-23*). The 2-D TLC method eliminates the need for the development of large numbers of plates in multiple solvent systems, reduces the amount of waste solvents for disposal, and substantially reduces the time required to identify active compounds. Bioautography has successfully driven the bioassay-guided fractionation of strawberry (*22*), *Artemisia drancunculus* (*24, 25*) and numerous other plant, algae, and marine invertebrates (*26, 27*).

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch012

Optimum Performance Laminar Chromatography (OPLC)

Direct bioautography coupled with OPLC offers numerous advantages for natural product isolation. Evaluation of interaction zones between fungi, bacteria, or seeds and biologically active natural products directly on the absorbent bed of the OPLC plate is a very powerful technique that will identify the presence of potential plant protectants, plant growth regulators, and anti-infective agents.

Tyihak *et al.* (*28*) first described OPLC as one of several forced flow planar chromatography techniques. Nyiredy (*29*) further described OPLC as a bridge between thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). OPLC offers an instrumental version of TLC that combines the advantages of TLC's planar layer with the automation and reproducibility of HPLC. Moreover, OPLC provides superior separation compared to conventional TLC, is faster than HPLC, and the sorbent bed of the planar layer is suitable for direct bioautography of a variety of biologically active compounds (*30-32*).

96-Well Microbiassay

Discovery, evaluation, and development of natural product fungicides are dependent upon the availability of miniaturized antifungal bioassays. A reference method [M27-A from the National Committee for Clinical Laboratory Standards (NCCLS)] for broth-dilution antifungal susceptibility testing of yeast was adapted for evaluation of antifungal compounds against sporulating filamentous fungi (*18*). This 96-well microtiter assay is used to determine and compare the sensitivity of fungal plant pathogens to natural and synthetic compounds with known fungicidal standards (*33*).

This standardized 96-well microtiter plate assay developed for discovery of natural product fungicidal agents is used to evaluate purified antifungal agents. A 96-well microtiter assay is used to determine sensitivity of *Colletotrichum acutatum, C. fragariae, C. gloeosporioides, Fusarium oxysporum, Botrytis cinerea, Phomopsis obscurans,* and *P. viticola* to the various antifungal agents in comparison with commercial fungicides. Fungicides such as benomyl, azoxystrobin and captan with different modes of action are used as standards in these assays. Each fungal species is challenged in a dose-response format so that the final test compounds concentrations of 0.3, 3.0 and 30.0 μM are achieved (in duplicate) in the different columns of the 96-well plate.

Fungal growth is evaluated by measuring absorbance of each well at 620 nm at 0, 24, 48, and 72 hr except for *P. obscurans* and *P. viticola*, where the data are recorded for up to 120 hr. Treatments are repeated so that mean absorbance values and standard errors can be calculated. Differences in spore germination and mycelial growth in each of the wells in the 96-well plate demonstrate sensitivity to particular concentrations of compound and indicate fungistatic or fungicidal effects.

A novel application of the microbioassay was also developed for the discovery of compounds that inhibit *Phytophthora* spp. This protocol used the 96-well format for high-throughput capability and a standardized method for quantification of initial zoospore concentrations for maximum reproducibility. Zoospore suspensions were quantifiable between 0.7 and 1.5×10^5 zoospores/mL using absorbance at 620 nm. Subsequent growth of mycelia was monitored by measuring absorbance at 620 nm at 24 hr intervals for 96 h. Full- and half-strength preparations of each of three media (V8 broth, Roswell Park Memorial Institute mycological broth, and mineral salts medium), and four zoospore concentrations (10, 100, 1000, and 10,000 zoospores/mL) were evaluated. Both full- and half-strength Roswell Park Memorial Institute mycological broth were identified as suitable synthetic media for growing *P.*

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch012

nicotianae, and 1000 zoospores/mL was established as the optimum initial concentration (*34*).

Detached Leaf Assay for Fungicide Evaluations *In Planta*

Anthracnose-susceptible 'Chandler' strawberry plants are grown in 10 x 10 cm plastic pots in a 1:1 (v/v) mixture of Jiffy-Mix (JPA, West Chicago, IL), and pasteurized sand in the greenhouse for a minimum of six weeks before inoculation. The plants are grown under standard conditions of a 16 hr day length and 24°C temperature. Growth parameters are varied as needed to accommodate needs of particular studies. Whole leaves (petiole and leaflets) are cut from plants no more than 4 hr before treatment or inoculation. Only the second or third youngest leaves on a plant are used for the fungicide assay, and only leaves with no visible signs of injury or symptoms of disease are collected. Immediately after collection, the leaves are placed in a tray lined with moist paper towels and the tray is closed to retain near 100% relative humidity (RH) and maintained at ~12°C. To test for protective fungicide activity, treatment compounds are applied to the upper surface of each of the three leaflets of the leaf using a chromatography sprayer. After treatment, the base of each leaf stem is inserted into sterile distilled water in a 100 x 10 mm tissue culture tubes. Each upper surface of each fungicide treated leaflet is then inoculated with the test fungal isolate within 24 hr of treatment. Inoculated leaves are subsequently incubated in a dew chamber for 48 hr at 100 % RH, 30°C and then maintained at 25°C in a moist chamber at 100 % RH for 10 days. Sterile distilled water is added to each tube as needed to maintain the surface of the water above the base of the petiole. If a compound is to be tested for curative activity (the ability of a fungicide to inhibit disease development after infection has occurred), the leaflets are inoculated 24 hr before the fungicidal compound is applied.

Experimental compounds are evaluated in a dose-response format. Azoxystrobin and other fungicides that have protective and curative activity are used as standards for comparison. A solvent control is used in each study. Each fungicide concentration is replicated four times, and the experiment is repeated at least once in time. This bioassay does not differentiate between direct effects on the fungus and indirect effects through induction of plant defenses. However, if a compound is much more active in this assay than when used only on the fungus in the microtiter assay, the latter type of activity is indicated.

This assay allows us to benchmark potential natural product lead compounds directly with a commercial standard (azoxystrobin) with a known mode of action (Q_o I inhibitor). The number of diseased lesions is used to determine effective concentrations needed for disease control. Lesion size is used to determine the relative effectiveness of the systemic activity that produces curative activity at 24 hr after inoculation.

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch012

Results and Discussion

Direct Bioautography

Bioautography techniques are the primary drivers of bioassay guided-fractionation in our laboratory. Matrix, one-dimensional, and two-dimensional bioautography protocols using *Colletotrichum* spp. as the test organisms have successfully led to the discovery of five promising new experimental antifungal compounds. Matrix bioautography is most often used to rapidly screen a large number of extracts for activity. Currently we are using matrix bioautography to initially evaluate a collection of 14,000 ascomycete and basidiomycete extracts for fungicidal activities. Matrix bioautography shows the location of two highly active antifungal zones out of 84 fungal extracts (Edward Mena, LifePharms, Inc., Groton, CT) applied to the 20 cm plate (Figure 1). While many extracts have fungal growth inhibitory activity, most fungal extracts are suppressive in nature, as indicated by the diffuse zone, and do not possess the sharp delineated clear zone of fungal growth inhibition that we are interested in.

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch012

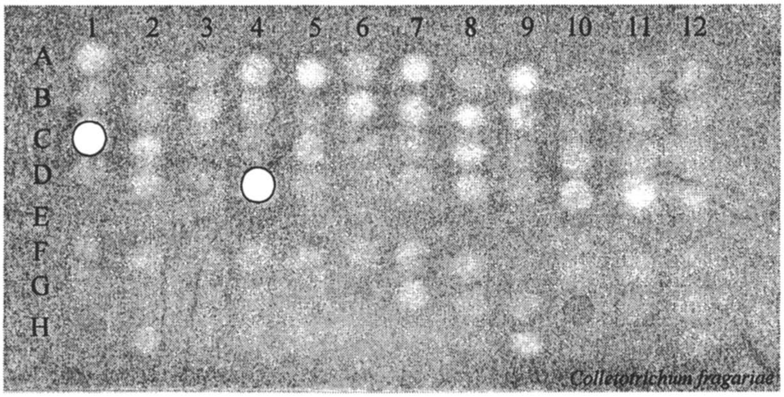


Figure 1. Matrix bioautography of 84 ethyl acetate extracts obtained from a library of ascomycete and basidiomycete fungi and reconstituted at 2 μg/100 μL in ethanol. Spots (zones) correspond to 5 μL aliquots applied to a silica gel TLC plate in an 8 row x 12 column format where row E served as a blank control. Due to the high level of activity of 1 μg fungicide standards (2 mM), they are spotted on a separate plate.

Once the most active extracts have been identified we then use 1-D TLC or 2D-TLC to further examine each active extract. Results from our bioautography studies suggest that chemically different populations of microorganisms and plants can easily be distinguished by their characteristic "2D-TLC fingerprint"

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch012

and antifungal zone patterns (*21*). Thus, this system represents a very useful technique for the identification and selection of chemically unique chemotypes from microbial isolates (*35*) and plant collections. Chromatographic properties such as relative polarity, UV absorbance, chemical reactivity associated with each active metabolite provide valuable information that can be useful in dereplication of known or nuisance compounds. When strains of different phytopathogenic fungi with dissimilar fungicide resistance profiles are inoculated onto replicate bioautography plates prepared from any given extract containing active metabolites, it is possible to visually observe distinct differences in the sensitivity of each fungal pathogen to single metabolites. These differences in pathogen sensitivity (fungicide resistance) can be observed by direct comparison of inhibition zone dimensions produced by active metabolites and control standards against each pathogenic strain tested. Chemical profiles can provide valuable information for rapidly selecting specific antifungal metabolites with unique activity against fungicide-resistant pathogens and can identify new compounds with potentially novel mechanisms of action. Bioautography has been used successfully to examine numerous extracts. Several examples demonstrating a wide range of fungistatic/fungicidal activities in plants are listed below.

While the mechanism of strawberry anthracnose resistance is unknown, we have evidence from bioautography that indicates that naturally occurring fungitoxic compounds are present in older leaves of strawberry. Our studies indicated that concentrations of fungitoxic compounds vary between anthracnose-resistant and anthracnose-susceptible cultivars and are present in different amounts in vegetative tissues of different ages (*22*). Using leaves of the anthracnose-susceptible cultivar Chandler and the anthracnose-resistant cultivar Sweet Charlie, we isolated and demonstrated the presence of three antifungal compounds. Results from this study indicate that anthracnose resistance in strawberry may depend on the concentration of two normally present antifungal compounds and the elicitation of a third compound in younger leaves. Two constitutive antifungal compounds are found in both 'Chandler' and 'Sweet Charlie' plants but 'Sweet Charlie' plants produced approximately 15 times more antifungal activity than 'Chandler' plants. Fungal growth inhibition associated with extracts from 'Chandler' plants appeared to be temporary. A third compound, detected exclusively in 'Sweet Charlie' plants, was produced only after young leaves were sprayed with a commercially available elicitor of antifungal compounds.

Thirty two naturally occurring quinones of four major classes: 1,4-naphthoquinones, 1,2-naphthoquinones, 1,4-benzoquinones, anthraquinones, and other miscellaneous compounds from our natural products collection were tested for antifungal activity using bioautography (*36*). Quinones demonstrated a good to moderate antifungal activity against *Colletotrichum* spp. *Colletotrichum fragariae* appeared to be the most sensitive species to quinone-based chemistry, *C. gloeosporioides* of intermediate sensitivity, and *C. acutatum* was the least sensitive species to these compounds.

Bioassay-directed isolation of antifungal compounds from an ethyl acetate extract of *Ruta graveolens* leaves yielded two furanocoumarins, one quinoline alkaloid, and four quinolone alkaloids, including a novel compound, 1-methyl-2-[6'-(3",4"-methylenedioxyphenyl)hexyl]-4-quinolone (*37*). Antifungal activities of the isolated compounds, together with 7-hydroxycoumarin, 4-hydroxycoumarin, and 7-methoxycoumarin which are known to occur in Rutaceae species, were evaluated by bioautography and microbioassay. Four of the alkaloids had moderate activity against *Colletotrichum* species, including a benomyl-resistant *C. acutatum*. These compounds and the furanocoumarins 5- and 8-methoxypsoralen had moderate activity against *Fusarium oxysporum*. The novel quinolone alkaloid was highly active against *Botrytis cinerea*. *Phomopsis* species were much more sensitive to most of the compounds, with *P. viticola* being highly sensitive to all of the compounds.

Methanol extract from the bark of *Macaranga monandra* inhibited fungal growth of *C. acutatum, C. fragariae, C. gloeosporioides, F. oxysporum, B. cinerea, P. obscurans* and *P. viticola* (*38*). Bioassay-guided fractionation led to the isolation of two active clerodane-type diterpenes that were elucidated by spectral methods (1D-, 2D NMR and MS) as kolavenic acid and 2-oxo-kolavenic acid. The 96-well microbioassay revealed that kolavenic acid and 2-oxo-kolavenic acid moderately inhibited growth of *Phomopsis viticola* and *Botrytis cinerea*.

CAY-1 is a fungicidal steroidal saponin (Figure 2, MW = 1243) isolated and identified by DeLucca *et al.* (*39*) from the ground fruit of cayenne pepper (*Capsicum frutescens*). CAY-1 was lethal to germinating conidia of *Aspergillus flavus*, *A. fumigatus*, *A. parasiticus* and *A. niger*. It was also active against agricultural and medicinally important fungi and yeast. *In vitro* dose-response assays with CAY-1 against plant pathogenic fungi showed that 3.0 µM inhibited growth of *C. gloeosporioides* and *C. acutatum* by 100% and *C. fragariae*, *P. obscurans*, and *P. viticola* by 90%. Detached leaf assays using strawberry leaves from anthracnose susceptible cv. Chandler demonstrated that CAY-1 decreased *C. fragariae* induced lesion number by 95% compared to the untreated control. Dose-response data indicated that CAY-1 appeared to be more active than azoxystrobin in disease control of *C. fragariae*-induced anthracnose leaf spot.

The application of the microbioassay to *Phytophthora nicotianae* was effectively used to determine EC_{50} values (*i.e.*, effective concentration for 50% growth reduction) for eight commercial antifungal compounds (azoxystrobin, fosetyl-aluminum, etridiazole, metalaxyl, pentachloronitrobenzene, pimaricin, and propamocarb). These EC_{50} values were compared to those obtained by conventional plate methods by measuring linear growth of mycelia on fungicide-amended medium. The microbioassay proved to be a rapid, reproducible, and

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch012

efficient method for testing the efficacy of compounds against *P. nicotianae* and should be effective for other species of *Phytophthora* as well. The assay requires relatively small amounts of a test compound and was suitable for the evaluation of natural product samples (*34*).

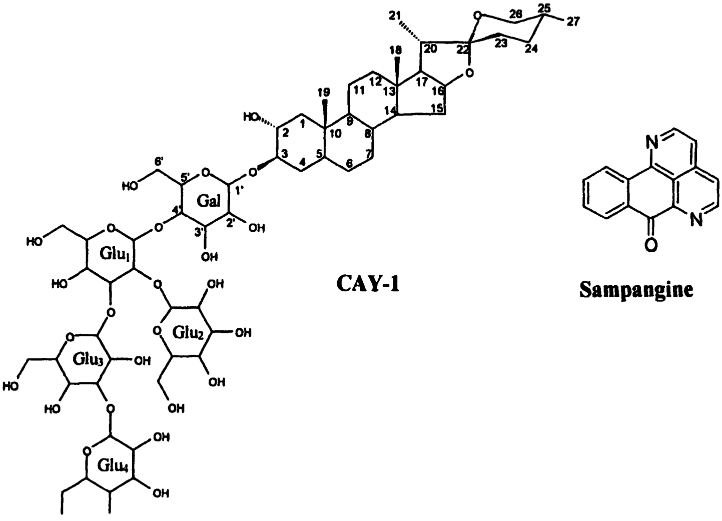


Figure 2. Structures of two experimental natural products with agrochemical potential as fungicides.

OPLC has provided us with a new tool to explore active extracts previously identified by matrix bioautography. OPLC has several advantages over TLC: enhanced reproducibility, greater separating power, shorter separating time, high sample throughput capability, enhanced 2-D analysis, and reduced spot/band broadening (*41*, Figure 3).

OPLC and bioautography of *Pimpinella* spp. essential oils demonstrated the presence of six antifungal metabolies active against three *Colletotrichum* species (Figure 4). Because of the OPLC's automation, a reference plate was prepared and available for direct comparison with the bioautography plates.

Detached leaf assays provide us with an opportunity to evaluate new fungicides directly on the leaf surface (Table 1). The detached leaf assay is also used to establish experimental field rates for future studies. Examination of the 'protectant' activity indicated that 1250 ppm CAY-1 and sampangine appeared to be effective concentrations for disease control of anthracnose on the leaf surface. This is between 100-1000 times the concentration required for in vitro activity of the fungus in liquid culture. The number of diseased lesions was used

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch012

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch012

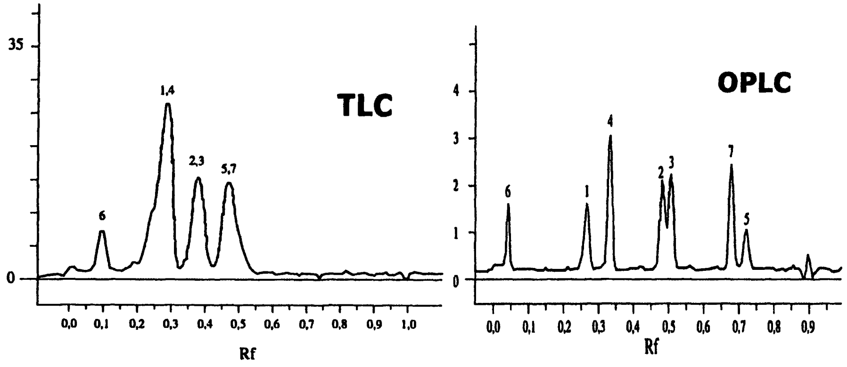


Figure 3. Densimetric analysis of nandrolone products by TLC and OPLC with 50% cyclohexane : 25% EtOAc : 25% $CHCl_3$ as the mobile phase. Figures are courtesy of BIONISIS SA, Le Plessis-Robinson, France.

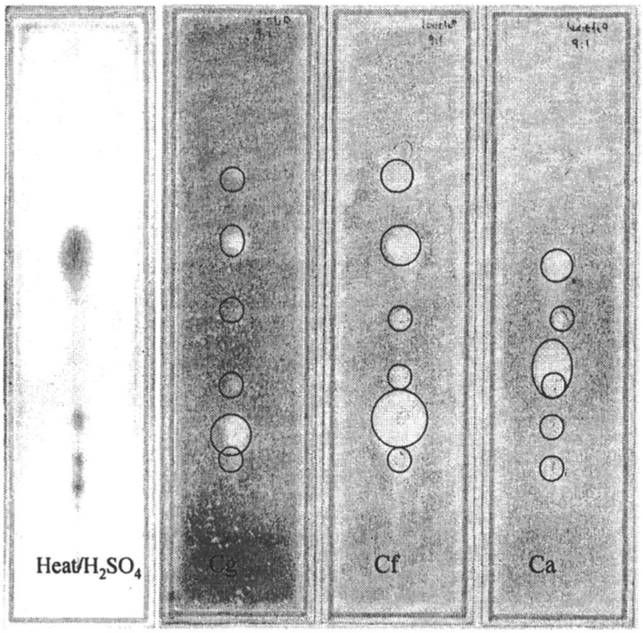


Figure 4. OPLC resolution and subsequent bioautography with Cg **(Colletotrichum gloeosporioides), Cf (C. fragariae), and Ca (C. acutatum)** *demonstrated six antifungal compounds in* **Pimpinella** *spp. essential oil and allowed for direct comparison with the H_2SO_4/heat reference TLC plate.*

to determine effective concentrations needed for disease control (Table 1, unpublished data by Wedge, Smith, and DeLucca). Lesion size was used to determine the relative effectiveness of the systemic activity that produced systemic-curative activity at 24 hr after inoculation (data not shown).

Table I. Number of leaf spots on detached leaves following inoculation with *Colletotrichum fragariae* isolate CF-75 and post treatment with commercial and experimental fungicides.

Concentration	N	Azoxystrobin		CAY-1		Sampangine	
ppm		Mean number of leaf spots/leaflet					
0*	45	22.34	a[z]	22.34	a	22.34	a
625	36	4.46	b	0.77	b	8.31	b
1250	36	1.58	b	0.35	b	1.26	c
2500	36	3.54	b	0.08	b	0.80	c
LSD[y]		5.97		4.99		5.54	

*Nontreated disease control.

[z]Means followed by the same letter are not significantly different.

[y]Least significant difference = LSD at $p<0.05$.

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch012

Conclusions

Knowledge from the results of evaluating thousands of extracts and pure compounds has culminated in the development of the standard operating protocol for natural product discovery that we currently use in our laboratory. Successful discovery, evaluation, and development of natural product fungicides are dependent upon the availability of high quality miniaturized antifungal bioassays. Bioassay-directed screening of compounds and extracts is the initial step in the discovery process for new agrochemicals and pesticides.

Standardization of inoculum allows for meaningful comparison of growth inhibition between different fungal pathogens, test compounds, and experiments repeated in time. Bioautography provides a simple technique to visually follow antifungal components through the separation process.

The 96-well microbioassay allows for the evaluation of microgram quantities, determination of dose-response relationships, and comparison of antifungal activity with fungicides of known mode of action. Coupling bioautography techniques with the 96-well microbioassay provides us with a discovery protocol that combines the simple and visual nature of direct bioautography with the rapid, sensitive, and high throughput capabilities of a microtiter system. The 96-well microbioassay is accurate and sensitive; as little as 0.1-1.0 μg amounts of test compound permit discrimination between germination and mycelial growth inhibitors and identification of fungicide resistant pathogens. The microbioassay utilizes a chemically defined liquid medium with a zwitterion buffer that limits chemical interaction with test compounds and controls pH. This new standardized method provides high-throughput capability with the capacity to study a chemical compound in detail, to perform mode of action studies, and to determine fungicide resistance profiles for specific fungal pathogens.

Detached leaf assays are critical to establishing some level of 'real world' activity prior to field testing that every agrochemical company wants before they invest the millions of dollars that are needed to develop a new agrochemical. Greenhouse efficacy testing ultimately helps determine the potential usefulness of compounds as pest control agents. To maximize the detection of natural products, high-throughput bioassay techniques must target significant agricultural pests, include relevant commercial pesticide standards, and adhere to sound statistical principles.

Allelochemicals and other natural product-based chemicals represent a rich new source of leads as agrochemicals. The identification of suitable natural product leads coupled with the traditional chemistry techniques should be an effective approach to optimize activity and specific chemical properties. Requirements for future fungicides to become valuable disease management tools are stringent, they must: be environmentally safe, efficacious at low rates, have new modes of action, and have a low to moderate risk of developing resistance in the target pathogen.

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch012

Disclaimer

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U. S. Department of Agriculture.

References

1. Wedge, D. E.; Camper, N. D. In *Biologically Active Natural Products: Agrochemicals and Pharmaceuticals*; H. G. Cutler, S. J. Cutler, Eds.; CRC Press. Boca Raton, FL. 2000 pp. 1-15. .
2. Agrios, G. N. In: *Plant Pathology*. Academic Press, San Diego, CA. 1997; pp. 63-82.
3. Oku, H. In: *Plant Pathogenesis and Disease Control*. Lewis Publishers. Boca Raton, FL, 1994; pp 64-69.

4. Vick, B. A.; Zimmerman, D. C. *Plant Physiol.* **1984,** *75,* 458-461.
5. Farmer, E. E.; Ryan, C. A. *Plant Cell* **1992,** *4,* 129-134.
6. Gundlach, H.; Muller, M. J.; Kutchan, T. M.; Zenk, M. H. *Proc. Natl. Acad. Sci. USA.* **1992,** *89,* 2389-2393.
7. Mueller, M. J.; Brodschelm, W.; Spannagl, E.; Zenk, M. H. *Proc. Natl. Acad. Sci. USA.* **1993,** 90, 7490-7494.
8. Bade, M. L.; Wick, R. L. *ACS Symp. Ser. 380,* American Chemical Society: Washington DC, 1988; pp 450-468.
9. Amborabe, E.; Aziz, A.; Trotel-Aziz, P.; Quantinet, D.; Dhuicq, L.; Vernet, G. *Phytoma* **2004,** *571,* 26-29.
10. Kobayashi, A.; Tai, A.; Kanzaki, H.; Kawazu, K. *Z. Naturforsh.* **1993,** *48,* 575-579.
11. Walker-Simmons, M.; Hadwiger, L.; Ryan, C. A. *Biochem. Biophys. Res. Commun.* **1983,** *110,* 194-199.
12. Wei, Z. *Amer. Chem. Soc. Abstr.* 225th meeting, **2003,** *225,* AGRO-071.
13. Carlin, C.; Faby, R.; Karjalainen, R.; Pommier, J. J.; Steffek, R. *Acta Horticulturae* **2004,** *649,* 237-240.
14. Kowalewski, A.; *Herger, G. Mededelingen van de Faculteit Landbouwwetenschappen, Univ.* **1992,** *52,* 449-456.
15. Inderjit; Nishimura, H. *Ann. Appl. Biol.* **1999,** *135,* 425-429.
16. Fuzellier, M. C.; Mortier, F.; Girard, T.; Payen, J. *Ann. Pharmaceutiques Francaieses* **1981,** *39,* 313-318.
17. Espinel-Ingrof, A.; Kerkering, T. M. *J. Clinic. Micro.* **1991,** *29,* 393-394.
18. Wedge, D. E.; Kuhajek, J. M. *SAAS Bull. Biochem. Biotech.* **1998,** *11,* 1-7.
19. Homans, A. L.; Fuchs, A. *J. Chromatogr.* **1970,** *51,* 327-3299.
20. Moore, R. E. *J. Indust. Microbiol.* **1996,** *16,* 134-143.
21. Wedge, D. E.; Nagle, D. G. *J. Nat. Prod.* **2000,** *63,* 1050-1054.
22. Vincent, A.; Dayan, F. E.; Maas, J. L.; Wedge, D. E. *Adv. Strawberry Res.* **1999,** *18,* 28-36.
23. Tellez, M. R.; Dayan, F. E.; Schrader, K. K.; Wedge, D. E.; Duke, S. O. *J. Agric. Food Chem.* **2000,** *48,* 3008-3012.
24. Meepagala, K. M.; Kuhajek, J. M.; Sturtz, G. D.; Wedge, D. E. *J. Chem. Ecol.* **2003,** *29,* 1771-1780.
25. Meepagala, K. M.; Sturtz, G. D.; Wedge, D. E. *J. Agric. Food Chem.* **2002,** *50,* 6989-6992.
26. Schrader, K. K.; Nagle, D. G.; Wedge, D. E. In *Advances in Microbial Toxin Research and its Biotechnological Exploitation*; Upadhyay, R. K., Ed.; Kluwer Academic/Plenum Publishers: New York, NY, 2002; pp. 171-195
27. Nagle, D. G. Wedge, D. E. In *Chemical Ecology of Plants: Allelopathy in Aquatic and Terrestrial Ecosystems.* Inderjit; Mallik, A, U., Eds.; Irkhauser Verlag: Switzerland, 2002; pp. 7-32.

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch012

28. Tyihak, E.; Mincsovics, E. In *Planar Chromatography: A retrospective view for the third millennium*. Sz. Nyiredy, Ed.; Springer Scientific Publisher: Budapest, 2001; pp. 137-176.
29. Nyiredy, S. *Trends Anal. Chem.* **2001**, *20*, 91-101.
30. Papp, E.; Farkas, A.; Otta, K.; Mincsovics, E. *J. Planar Chromatogr.* **2000**, *13*, 328-332.
31. Galand, N.; Pothier, J.; Viel, C. *J. Chromatog. Sci.*, **2002**, *40*, 585-597.
32. Tyihak, E.; Botz, L.; Ott, P.; Nagy, S.; Kocsis, B.; Kiraly-Veghely, Z.; Mincsovics, E. *Chem. Anal.* 2003, *48*, 543-553.
33. Wedge, D. E; Galindo, J. C. G.; Marcias, F. A. *Phytochemistry* **2000**, *53*, 747-757.
34. Kuhajek, J. M.; Jeffers, S. N.; Slattery, M.; Wedge, D. E. *Phytopathology* **2003**, *93*, 46-53.
35. Nagle, D. G.; Paul, V. J. *J. Phycol.* **1999**, *35* (*Suppl. 607*),1412-1421.
36. Meazza, G.; Dayan, F. E.; Wedge, D. E. *J. Agric. Food Chem.* **2003**, *51*, 3824-3828.
37. Oliva, A.; Meepagala, K. M.; Wedge, D. E.; Hale, A. L.; Harries, D.; Aliotta, G.; Duke, S. O. *J. Agric. Food Chem.* **2003**, *51*, 890-896.
38. Salah, A. M.; Bedir, E.; Ngeh, T. J.; Kahn, I. A.; Wedge, D. E. *J. Agric. Food Chem.* **2003**, *51*, 7607-7610.
39. De Lucca, A. J.; Bland, J. M.; Vigo, C. B.; Cushion, M.; Selitrennikoff, C. P.; Peter, J.; Walsh, T. J. *Med. Mycol.* **2002**, *40*, 131-137.
40. Wedge, David E.; Nagle, Dale G. US 2004192721, 2004.
41. Bryson, N.; Papillard, D. *LCGC North America* **2004**, (Suppl.), 88-94.

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch012

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch013

Chapter 13

Natural Insect Repellents: Activity against Mosquitoes and Cockroaches

Gretchen Schultz[1], **Chris Peterson**[1,2], **and Joel Coats**[1,*]

[1]Department of Entomology, Iowa State University, 112 Insectary, Ames, IA 50011
[2]Current address: Wood Products Insect Research Unit, Forest Service, U.S. Department of Agriculture, Starkville, MS 39759

Recent research has focused on the repellent properties of extracts from the catnip plant (*Nepeta cataria*) and the Osage orange (*Maclura pomifera*) fruit. This chapter includes results on German cockroach (*Blattella germanica*), and house fly (*Musca domestica*) contact irritancy to catnip essential oil, and its major components, *Z,E*-nepetalactone and *E,Z*-nepetalactone, compared with the commercial standard, N,N-diethyl-*m*-toluamide (DEET). Both species showed high percentage repellency values when exposed to filter paper treated with catnip essential oil or the individual nepetalactone isomers. Of the two nepetalactone isomers evaluated, German cockroaches were most responsive to the *E,Z* isomer. House flies showed similar trends in contact irritancy, responding to surfaces treated with the predominant catnip isomer, *Z,E*-nepetalactone, more intensely than to the catnip essential oil. Catnip and Osage orange essential oils, and a sesquiterpene found in Osage orange, elemol, were evaluated for repellency to the northern house mosquito (*Culex pipiens*) and are presented here. Two mosquito bioassays were used to measure percentage and contact repellecy. Mosquitoes responded initially with high percentage repellency to surfaces treated with catnip essential oil. From the residual repellency

In Natural Products for Pest Management; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

study, this trend in repellency by the catnip oil significantly decreased over the 180-minute test period. Elemol, and DEET initially had lower percentage repellency values than catnip essential oil, but did not show the negative relationship between percentage repellency and time, retaining excellent repellency throughout the 3-hour bioassay. Solutions with elemol and DEET exhibited greater significance in contact repellency compared to catnip essential oil. These results show that catnip essential oil is a potent mosquito repellent, but does not provide the same residual effects as the commercial standard, DEET. Elemol, a sesquiterpene extracted from the fruit of the Osage orange, shows excellent promise as a mosquito repellent with comparable activity to DEET in contact and residual repellency.

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch013

Over the last 20 years there has been intensive effort toward the development of natural products in pest control. Much of this initiative is due to increased regulations on the use of chemicals in insect pest management. Consumers have shown increased interest in and support for products that are safer to human health and more environmentally friendly than many of the traditional chemicals with high acute toxicity and long-lasting residues. Naturally derived biorepellents have been investigated as a group of chemicals that have biological activity and can cause repellent or insecticidal effects without negative impacts on human safety and the environment.

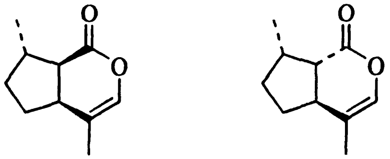
Some of the more common chemicals that have historically been used as mosquito repellents include dimethyl phthalate (DMP), 2-ethyl-1,3-hexanediol (Rutgers 612), dimethyl carbate, benzyl benzoate, butyl 3,4-dihydro-2,2-dimethyl-4-oxo-2H-pyran-6-carboxylate (Indalone), and N,N-diethyl-*m*-toluamide (DEET), which is currently the most widely used and effective mosquito repellent available. Several reports on DEET toxicity, citing encephalopathy in children, anaphylaxis, urticaria syndrome, and hypotension (*1, 2, 3, 4*), have intensified the initiative for developing alternative insect repellents. In recent years, several botanical insect repellents have become available on the market and most commonly include components from at least one of the following extracts: citronella, cinnamon, cedar, eucalyptus, mints, lemongrass, geranium, and soybean (*5*). Neem oil, an extract of the Neem tree, *Azadirachta indica*, is another natural product that has shown repellency of *Anopheles* mosquitoes (*6*).

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch013

Many plant oils and extracts have been identified as insect deterrents, repellents or toxins. In addition to economic disadvantages holding back the commercialization of some natural products, one underlying limitation with these botanical materials is that many of them do not offer residual control equivalent to synthetic standards like DEET (*5*). Research in the Pesticide Toxicology Laboratory at Iowa State University has focused on the identification of compounds present in the extracts of two plants, the Osage orange (*Maclura pomifera,* Moraceae) and catnip (*Nepeta cataria,* Lamiaceae). Recent emphasis has been placed on understanding the mechanism of repellency and developing natural products that can offer increased potency and/or residual repellency.

Catnip

Catnip (*Nepeta cataria*) is an herbaceous mint native to Eurasia and North Africa. Its present distribution includes most of North America, with great wild abundance around the Great Lakes, and commercial production in Alberta, British Columbia, Alaska, Washington, Oregon, and California. The first uses of catnip for insect control are referenced in folklore. Over the past 50 years, experiments have validated its insect repellent activity (*7, 8*). Nepetalactone, the active ingredient present in catnip plant extracts, is known to occur as two isomers: *Z,E* and *E,Z*-nepetalactone *(9*). These two diastereomeric isomers are structurally very similar and differ only in the orientation of substituents across one bond. Past efforts from our lab analyzed the comparative repellency of these nepetalactone isomers. One particular study conducted previously on the German cockroach (*Blattella germanica*) is included in this report.



Z,E- nepetalactone E,Z- nepetalactone

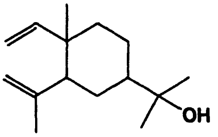
Figure 1. Z,E and E,Z nepetalactone isomers in catnip.

Osage Orange

The Osage orange is another source for natural products with insect repellent properties. *Maclura pomifera*, the osage orange or hedge apple tree, was used by early pioneers in the Midwest for dyes, the wood was used in bow making, trees were planted to create hedge rows (which served as living fences and windbreaks), and fruits were reportedly useful in repelling insects and

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch013

spiders. Settlers placed whole fruits in their cupboards to ward off spiders, roaches, and other pests (*10*). Early studies on extracts of the Osage orange fruit focused on effects of two isoflavones, osajin and pomiferin (*11*), and five components of the essential oil obtained by steam distillation (*12*). Elemol, one of the major components of the essential oil, is a sesquiterpene alcohol. This compound has shown significant repellency to several species of insects in our laboratory studies, some of which are presented in this report.



Elemol

Figure 2. Elemol, a sesquiterpene alcohol present in the essential oil of the Osage orange (Maclura pomifera).

Repellency Bioassay Methods

German Cockroach and House Fly Bioassay

A choice-test arena was used to assess irritancy of test solutions to two common household insect pests, the German cockroach (*Blattella germanica*) and the house fly (*Musca domestica*). Catnip essential oil obtained by steam distillation, and the two major components of its essential oil, *Z,E*-nepetalactone and *E,Z*-nepetalactone (isolated from the essential oil by preparative TLC), were evaluated for behavioral effects of **contact irritancy** to the German cockroach (*9*); catnip essential oil and its major constituent *Z,E*-nepetalactone were tested against the house fly. N,N-diethyl-*m*-toluamide (DEET) (Aldrich, St. Louis, MO) served as a positive control for the choice-test arena assay and as a point of comparison for measuring insect behavioral effects that result from current commercial insect repellents. Test solutions ranging from 10% to 0.1% (vol/vol) active ingredient (a.i.) were made up in acetone and then delivered on to a filter paper for solvent evaporation. Resulting rates of a.i. were 1.63 mg/cm^2, 815$\mu g/cm^2$, 163$\mu g/cm^2$, 81.5$\mu g/cm^2$, and 16.3$\mu g/cm^2$. Choice-test arenas for German cockroaches and house flies were constructed from plastic Petri dishes. One-half of a 12.5-cm dia. filter paper was treated with 1 ml test solution, and the other was treated with 1 ml of only solvent (control). Both halves of the filter paper were placed in the choice-test arena. Position of the treated filter paper was randomized using a random-number table. Individual German cockroaches or house flies were placed in each choice-test arena through a centered hole in the lid of the Petri dish and evaluated for a 300-second period. The amount of time the insect spent on the treated and untreated filter papers were recorded and used to calculate a "percentage repellency" value:

Percentage Repellency = ((Time on Untreated - Time on Treated)/ 300) x 100

Ten replicates of each treatment solution were tested for both German cockroaches and house flies. Details of this assay design and some results have previously been described (*8, 13*).

Mosquito Repellency

Insects

A colony of *Culex pipiens,* 10 generations removed from wild mosquitoes collected in Ames, Iowa, was used for testing. The colony was blood-fed on the bobwhite quail, *Colinus virginianus*. Eggs from mosquitoes were dried and stored in an incubator until needed. Eggs were placed in deoxygenated water and two to three drops of a ground TetraMin™ fish food solution were added to the water to feed the larvae. Pupae were removed from the larval pans as they appeared and were placed into mesh-covered paper cups. Following emergence, adult females were tested over a six-day period. The mosquitoes were continually allowed to feed on a cotton ball soaked with 0.3 M sucrose solution. At 1-2 hours before testing began, the cotton balls were removed, and the mosquitoes were preconditioned in the bioassay environmental chamber, held at 26°C, for 1-2 hours.

Percentage and Contact Repellency Bioassay

A static-air choice-test apparatus was used to determine the behavioral effects on the insects in this study. The apparatus consisted of a 9 x 60-cm section of glass tubing with a 2-cm hole drilled at the midpoint along the length for central introduction of the insects. All of the testing was conducted in an environmental chamber at 26°C. Treatments included catnip essential oil, obtained by steam distillation previously described by Peterson et al. 2002 (*9*), Osage orange essential oil, obtained by steam distillation of whole fruits previously described by Peterson 2002 (*13*), elemol (Augustus Oils, New Hampshire, England), and DEET (Aldrich, St. Louis, MO) test solutions at 1%, 0.5% and 0.1% concentrations (wt/vol). The test solutions' solvent, hexane, served as a control treatment in this assay. One milliliter of the solution was applied to one half of a 9-cm diameter round filter paper with an area of 63.6

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch013

cm^2 and then allowed to dry before testing. This resulted in the following rates of exposure: 157, 78.6 and 15.7 $\mu g/cm^2$. Treated filter papers were placed inside the lids of 9-cm glass petri dishes, and placed over the ends of the glass tube. The position of the treated side, to the right or to the left, was selected by using a random-number table. Approximately fifteen unmated adult female mosquitoes were anaesthetized with CO_2 and then introduced to the 9 x 60-cm glass cylinder through the centered 2-cm hole. Timing began 2 minutes after mosquito introduction, and mosquito distribution inside the static-air choice-test apparatus was observed over a 180-minute period for each treatment. Mosquito distribution (number of individuals on treated and untreated side) was recorded at 15, 30, 60, 90, 120, and 180-minute timpoints. The data generated by this study was used to examine two measures of mosquito repellency, "percentage repellency" and "contact repellency." Percentage repellency was calculated for with the following formula:

Percentage Repellency = ((Number of Individuals on Untreated Half - Number of Individuals on Treated Half)/15) x 100

Contact repellency was defined in this assay as 100% avoidance of the treated filter paper (no contact). 15, 30, 60, 90, 120, and 180-minute time-points were used to assess contact repellency for individual observations.

The experimental design was a completely randomized design using three replications of each treatment. Analysis of variance was performed on SAS (PROC GLM; SAS Version 8) to identify significant differences of percentage repellency due to treatment, and concentration. Multiple comparisions were completed using Tukey's procedure. Treatment pair-wise comparisons of contact repellency, which included data from the six time-points observed for each treatment, were completed using Fishers Exact (PROC FREQ; SAS Version 8).

Mosquito Residual Repellency Bioassay

Aged applications of catnip essential oil, elemol, DEET, and hexane (control) were compared in the static-air choice-test apparatus under the same conditions as described above. The 0.5% and 0.1% (wt/vol) solutions of each test solution were made to yield the same rate of a.i. used in the above mosquito repellency bioassay. Individually treated filter papers were then placed in a fume hood and aged for 0, 30, 60, 120, or 180-minutes, allowing volatization to occur over a set period of time. After the specified ageing period, filter papers were placed on the inside of the 9-cm glass petri dish lids, and then placed over the ends of the glass tube. The position of the treated side was randomized.

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch013

Approximately 18 unmated adult female mosquitoes were anaesthetized with CO_2 and then introduced to the 9 x 60-cm glass cylinder through the centered 2-cm hole. Timing began 2 minutes after mosquito introduction, and mosquito distribution (number of individuals on treated and untreated sides) inside the static-air choice-test apparatus was recorded after 15 minutes for determination of Percentage Repellency (calculations shown under Percentage and Contact Repellency Bioassay). Experimental design was completely randomized with three replications of each aged test solution. Analysis of variance was used to identify significant differences related to a.i., concentration, and ageing period. Regression analysis was used to examine percentage repellency relationship to filter paper ageing.

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch013

Results

German Cockroach and House Fly Repellency

The German cockroach and house fly both showed contact irritancy responses to at least one concentration of each test solution evaluated (Table I). German cockroaches gave the highest percentage repellency value response when exposed to the 0.5% solution of *E,Z*-nepetalactone. This percentage repellency response was more than four times the response seen from testing the same concentration of *Z,E*-nepetalactone. In the cockroach experiment, both *Z,E* and *E,Z*-nepetalactone isomers caused an overall higher percentage repellency response at lower concentrations of the respective a.i., compared to treatments with DEET. The house fly responded to the test solutions with a similar trend, although the *E,Z* isomer was not tested. The higher percentage repellency values resulted from exposure to catnip essential oil and to *Z,E*-nepetalactone, ranging from 70-96%, compared to DEET (39%) (Table II).

Mosquito Repellency

Percentage repellency of catnip and osage orange essential oil, elemol and DEET at 15 minutes is represented in Figures 3, 4, and 5. All compounds tested showed various levels of significance in percentage repellency and contact repellency. The overall concentration effect was not significant ($P = 0.4569$). Osage orange essential oil represented the lowest values in percentage repellency (<60%) and did not show any significant contact repellency ($P = 0.1$). Catnip essential oil showed high percentage repellency at the 15-minute time-point at all concentrations tested, including the highest value, 100% from the 0.1% concentration (Figure 5). This was also the most significant level of contact repellency ($P < 0.0001$) resulting from the three concentrations of catnip essential

Table I. Percentage repellency of catnip essential oil, *Z,E*-nepetalactone, *E,Z*-nepetalactone, DEET and control to the German cockroach, *Blattella germanica*, in the choice-test arena bioassay*.

Treatment	*Application Rate*	*Percentage Repellency ± SEM*
Controls	Acetone	5.2 ± 7.5a
	Hexane	2.9 ± 3.7a
DEET	1.60 mg/cm^2	58.3 ± 10.5b
	800 µg/cm^2	25.8 ± 9.5a
	160 µg/cm^2	20.4 ± 9.2a
	80 µg/cm^2	15.5 ± 5.4a
Catnip Essential Oil	800 µg/cm^2	55.6 ± 9.8b
	160 µg/cm^2	27.7 ± 13.1ab
	80 µg/cm^2	33.7 ± 15.7ab
Z,E-Nepetalactone	800 µg/cm^2	68.2 ± 5.7b
	160 µg/cm^2	56.8 ± 7.8b
	80 µg/cm^2	15.4 ± 6.9a
	16 µg/cm^2	16.1 ± 7.4a
E,Z-Nepetalactone	80 µg/cm^2	79.4 ± 3.5c
	16 µg/cm^2	46.4 ± 11.0b

*Treatments with the same letter are not signigicantly different by least-squares means analysis at α = 0.05 (8).

SOURCE: Reproduced with permission from reference 8. Copyright 2002.

Table II. Percentage repellency of DEET, catnip essential oil, *Z,E*-nepetalactone, and control to the house fly, *Musca domestica*, in the choice-test arena bioassay (*13*).

Treatment	*Application Rate*	*Percentage Repellency*
Control	-	-5.3
DEET	800 µg/cm^2	20.7
	160 µg/cm^2	19.3
	80 µg/cm^2	38.7
Catnip Essential Oil	80 µg/cm^2	63.3
	160 µg/cm^2	70.0
	80 µg/cm^2	52.7
Z,E-Nepetalactone	800 µg/cm^2	96.0
	160 µg/cm^2	69.3
	80 µg/cm^2	87.3

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch013

oil (Table III). The other concentrations of catnip essential oil varied in contact repellency (0.5% concentration, P = 0.5, and 1% concentration, P = 0.02). Elemol solutions yielded the second highest set of percentage repellency values of the test solutions, ranging from 81% to 63%. These treatments also resulted in highly significant contact repellency (Table III). The commercially available standard for mosquito repellency, DEET, also showed high percentage repellency values, ranging from 63% to 44%, in addition to high significance for contact repellency.

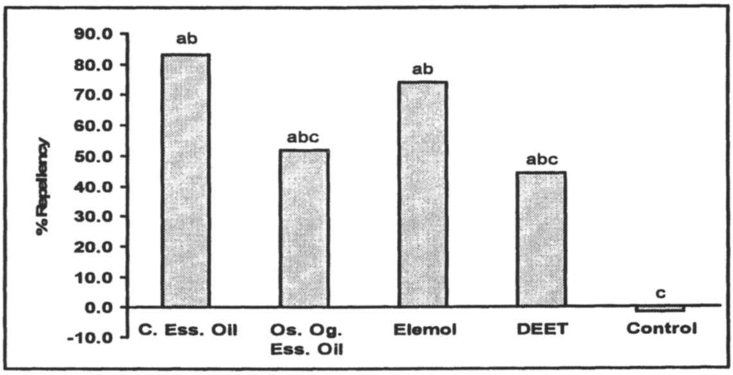


Figure 3. 15-minute percentage repellency of the northern house mosquito, Culex pipiens, *in a static-air repellency chamber to 157 μg/cm² application (1% concentration) of catnip essential oil, elemol, DEET, as well as osage orange essential oil, and a solvent control. Treatments with the same letter are not significantly different by Tukey analysis at α = 0.05.*

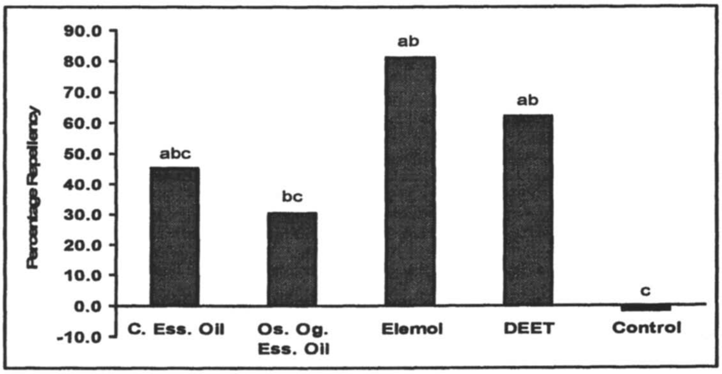


Figure 4. 15-minute percentage repellency of the northern house mosquito, Culex pipiens, *in a static-air repellency chamber to 78.6 μg/cm² application (0.5% concentration) of catnip essential oil, elemol, DEET, as well as osage orange essential oil, and a solvent control. Treatments with the same letter are not significantly different by Tukey analysis at α = 0.05.*

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch013

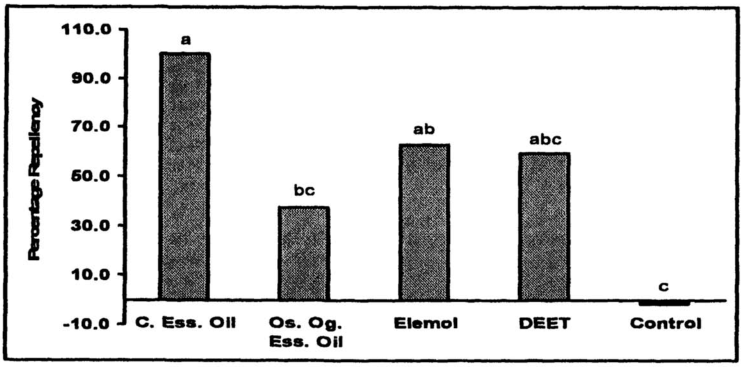


Figure 5. 15-minute percentage repellency of the northern house mosquito, Culex pipiens, *in a static-air repellency chamber to 15.7 μg/cm² application (0.1% concentration) of catnip essential oil, elemol, DEET, as well as osage orange essential oil, and a solvent control. Treatments with the same letter are not significantly different by Tukey analysis at α = 0.05.*

Table III. Contact repellency of the northern house mosquito, *Culex pipiens*, measured at 15, 30, 60, 90, 120, 180-minutes in a static-air repellency chamber to catnip essential oil, Osage orange essential oil, elemol, DEET, and control.*

Treatment	*Application Rate*	*Treatment vs. Control P value*
Catnip Essential Oil	157 μg/cm²	0.02
	78.6 μg/cm²	0.5
	15.7 μg/cm²	<0.001
Osage Orange Essential Oil	157 μg/cm²	0.1
	78.6 μg/cm²	0.5
	15.7 μg/cm²	0.5
Elemol	157 μg/cm²	<0.001
	78.6 μg/cm²	<0.001
	15.7 μg/cm²	<0.001
DEET	157 μg/cm²	<0.001
	78.6 μg/cm²	<0.001
	15.7 μg/cm²	<0.001
Control	-	-

* *P*-values in the table are from Fisher Exact test.

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch013

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch013

Residual Repellency

Percentage repellency values were high for catnip essential oil, elemol, and DEET solutions immediately following application to the test surface (Table IV). The analysis of variance showed that there was a difference among the three different solutions and the control ($P < 0.0001$), and a significant interaction with treatment solution and time ($P = 0.0019$). The only treatment solutions to show a significant decrease in percentage repellency over time were 0.5% catnip essential oil ($P = 0.02$) and 0.1% catnip essential oil ($P = 0.003$) in which 51% of the variability in the data was explained by this negative linear relationship. Elemol, DEET, and control treatments did not show significant trends in the regression analysis, indicating maintenance of repellency with elemol and DEET over the 3-hour period.

Table IV. Residual percentage repellency of the northern house mosquito, *Culex pipiens*, to 0, 30, 60, 90, 120, 180-minute aged treatments of 0.5% and 0.1% solutions of catnip essential oil, elemol, DEET, and control in a static-air repellency chamber.

Treatment	Application Rate	*Percentage Repellency Over Time*				
		0 min	*30 min*	*60 min*	*120 min*	*180 min*
Catnip Essential Oil	78.6 μg/cm^2	71.5	88.6	59.8	24	31.9
	15.7 μg/cm^2	88.8	37	40.7	22.2	7.4
Elemol	78.6 μg/cm^2	84.7	76.5	96.5	80.8	76.5
	15.7 μg/cm^2	35.0	30.8	49	20.7	44.8
DEET	78.6 μg/cm^2	74.0	37	59	77.7	74
	15.7 μg/cm^2	54.9	23.1	45.7	39	70.6
Control	-	-6.1	-9.3	1.3	25.5	-9.1

Conclusions

Bioassays in a choice-test arena were used to assess cockroach and house fly irritancy responses. The use of deterents is a valuable tool for pest control, particularly when used with an integrated pest management program. In the studies we report, contact irritancy serves as a measure of deterrence and helps to identify compounds that may serve as effective protectants for premises. It should be noted that limitations of this method are that individuals are only exposed to the treated surface for a 5-minute period and can only characterize a short-term response.

German cockroaches and house flies responded negatively to all solutions evaluated. These results demonstrate the efficiency of the assay and add support for catnip essential oil as an insect repellent. Specifically, cockroaches showed greatest avoidance of filter papers treated with the purified nepetalactone isomers, *Z,E* and *E,Z,* and house flies showed greatest avoidance of *Z,E*-nepetalactone. Both nepetalactone isomers were compared during trials on the German cockroach, and the result was a much higher percentage repellency from papers treated with *E,Z*-nepetalactone. These results raise the need for structure-activity relationship studies, since *Z,E*-nepetalactone and *E,Z*-nepetalactone are very similar compounds that only differ in orientation of groups across one bond on the molecule. Additional studies on the mode of action of deterrents are required before conclusions are drawn on how the minimal structural difference in *Z,E* and *E,Z*-nepetalactone cause significantly different responses from *B. germanica.*

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch013

Initial investigations of mosquito repellency with catnip and osage orange essential oil allowed us to directly compare with DEET, the current commercial standard, and further analysis helped identify differences in the activity of these compounds as insect repellents. At present, there is no one characteristic that fits all repellents or a single mechanism that explains how specific chemicals and blends act on insects. Studies have shown that an insect's response to the chemicals in the environment is dependent on their physiological and developmental state (*14*). The studies presented in this report focus on adult female mosquitoes and their responsiveness to various rates of catnip and Osage orange essential oil, elemol, and DEET over time. Results from mosquito repellency assay show that after 15-minutes, the northern house mosquito was most significantly repelled from the filter paper surfaces treated with catnip essential oil (100%). The percentage repellency values from the DEET and elemol treatments resulted in a lower range (81%-44%) than catnip essential oil, but showed higher contact repellency. Observations during the assay showed that individuals exposed to catnip essential oil moved further away from the treated surface than in the DEET and elemol treatments. Over time, this effect started to decrease with catnip essential oil as mosquitoes redistributed through the tube, eventually reaching a distribution similar to the control

Mosquitoes exposed to DEET and elemol settled far enough from the treated surface to achieve an adequate level of contact repellency. As time increased, individuals would continually reject the treated surface up to the end of the 180-minute period, unlike the catnip essential oil, which exhibited an initially high repellency response that decreased over time. DEET and elemol showed a longer duration of repellency compared to catnip essential oil, as is evidenced with higher significance in contact repellency. Additional studies are needed to better understand how these differences occur, including studies on the chemical volatilization, and interference with behavioral stages of mosquito host-finding and acceptance.

The second mosquito assay focused on quantifying the residual repellency of the northern house mosquito to aged filter papers of catnip essential oil, elemol and DEET. All 0.5% and 0.1% test solutions showed significant percentage repellency following application (i.e., with no ageing period). This repellency effect slowly decreased over time for both concentrations of catnip essential oil (0.5%, P=0.02, 0.1%, P=0.003). There was no significant loss in percentage repellency seen in the DEET and elemol treatment solutions, accounting for continual mosquito repellency over 3 hours from a treated surface. Olfactory repellency differs from contact repellency, and the method used here allows for some differentiation between the two types. The high initial repellency of catnip essential oil is not sustained over a 3-hour period, but elemol and DEET do show residual repellency to that time-point.

The series of experiments presented here give supporting evidence for catnip and Osage orange essential oils, elemol, and nepetalactone as effective insect repellents to common household pests and pests of human health. Investigations with mosquito behavioral responses in a static-air apparatus showed that catnip essential oil, and elemol can act as effective mosquito repellents from treated surfaces, but differ in residual efficacy. Further studies are currently underway to evaluate residual repellency effects of other natural products in Osage orange essential oil and examine differences in the mechanism of repellency.

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch013

Acknowledgements

We appreciate the assistance from Dr. Wayne Rowley and Brad Tucker for providing the mosquitoes for the studies. We thank Dianna Wilkening, Theresa Redding, and Melinda Thede for their help with insect rearing and conducting bioassays. Contents of this paper were presented at the 226th National American Chemical Society Meeting, Anaheim, CA, March 28, to April 1, 2004.

References

1. Miller, J. D. *N Engl J Med.* **1982**, 307, 1341-2.
2. Veltri, J. C.; Osimitz, T. G.; Bradford, D. C.; Page, B. C. *J. Toxicol. Clin. Toxicol.* **1994**, 32, 1-16.
3. von Mayenburg, J.; Rakoski, J. *Contact Dermatitis.* **1994**, 4, 9, 171.
4. Zadikoff, C. M. *J. Pediatr.* **1979**, 95, 140-2.
5. Fradin, M. S.; Day, J. F. *N. Engl. J. Med.* **2002**, 347, 13-18.
6. Sharma, V. P.; Ansari, M. A.; Razdan, R. K. *J. Am. Mosquito Cont.* **1993**, 9, 359-360.
7. Eisner, T. *Science.* **1964**, 146, 1318.
8. Peterson, C. J.; Nemetz, L. T.; Jones, L. M.; Coats, J. R. *J. Econ. Entomol.* **2002**, 95, 377-380.
9. McElvain, S. M.; Bright, R. D.; Johnson, P. R. *J. Am. Chem. Soc.* **1941**, 63, 1558-1563.
10. Sand, S. *Am. Hortic.* **1991**, 70, 37.
11. Peterson, C. J.; Fristad, A.; Tsao, R.; Coats, J. R.; *Environ. Entomol.* **2000**, 29, 1133-1137.
12. Peterson, C. J.; Zhu, J.; Coats, J. R. *J. Essent. Oil Res.* **2002**, 14, 233-236.
13. Schultz, G.; Simbro, E.; Belden, J.; Zhu, J.; Coats, J. R. *Environ. Entomol.* **2004**, 33, 1562-1569.
14. Klowden, M. J.; Davis, E. E.; Bowen, M. F. *J. Insect Physiol.* **1987**, 33, 643-646.

Downloaded by PENNSYLVANIA STATE UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch013

Downloaded by STANFORD UNIV GREEN LIBR on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch014

Chapter 14

Insect Antifeedant Activity of Natural Products and the Structure–Activity Relationship of Their Derivatives

Masanori Morimoto and Koichiro Komai

Department of Agricultural Chemistry, Faculty of Agriculture, Kinki University, Nakamachi 3327–204, Nara 631–8505, Japan

Many plants have chemical defense systems to avoid attack from phytophagous insects and phytopathogens. These natural products act as repellents and antifeedants against phytophagous insects, and include all categories of natural products (*e.g.* terpenes, alkaloids, quinones, phenolics). Dihydrobenzofuran, remirol and cyperaquinones produced by Cyperaceae, showed insect antifeedant activity against *Spodoptera litura* larvae. Similarly, polymethylflavonoids produced by *Gnaphalium affine* (Asteraceae) showed antifeedant activity against the same insect. Dihydrobenzofuran derivatives were prepared using known coupling reactions using phenols and dibromo-2-methyl-2-butene. Structureactivity relationship (SAR) studies of these derivatives suggested that introduction of acetyl and methoxyl groups to the benzene rings increased biological activity. The most effective derivative was 7-acetyl-4,6-dimethoxy-2,3-dihydrobenzofuran (ED_{50}: 5.4 nmol/cm^2 against *S. litura* 3rd instar larvae). On the other hand, SAR of flavonoids including chromone, suggested that high melting point and a hydroxyl group substituent decreased the biological activity. Some chromones (*e.g.*, 2-methyl-5,6,7-trimethoxychromone: ED_{50} 100 nmol/cm^2 against *S. litura* 3rd instar larvae) were more effective than the active flavones in this bioassay.

In Natural Products for Pest Management; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

Downloaded by STANFORD UNIV GREEN LIBR on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch014

Introduction

Plants can not move their habitat easily, so mechanisms of adaptaion to environmental conditions developed with biological selection. Consequently, a large mumber of secondary metabolites were produced by various complex biosynthetic pathways in the plant kingdom (*1*). It is well known that some of these compounds act as defense against phytophagous insects and phytopathogens. Defense chemicals such as insect antifeedants, insecticidal compounds, and repellents have various modes of action (2). Natural products have been used as pest control agents by ancient civilizations. These days, these biologically active natural products are used as lead compounds in the development of agrochemicals (*3*). Physostigmine is an alkaloid found in the seed of ***Physostigma venenosum*** (Leguminosae). It was a lead compound for the carbamate insecticides (*4*) (Figure 1). Natural pyrethroids are insecticidal terpenoids produced by the pyrethrum flower, *Chrysanthemum cinerariaefolium* (Asteraceae) (*5*). Nicotine is a major alkaloid produced by tobacco, *Nicotiana rustica* (Solanaceae) and is a non-specific neuro-toxic compound against both mammals and insects. However, the neonicotinoid insecticides, for which nicotine was the lead compound, have high specific toxicity against insects (*6*). The goal of screening phytochemicals is to discover new chemicals and new modes of action for development of new agrochemicals.

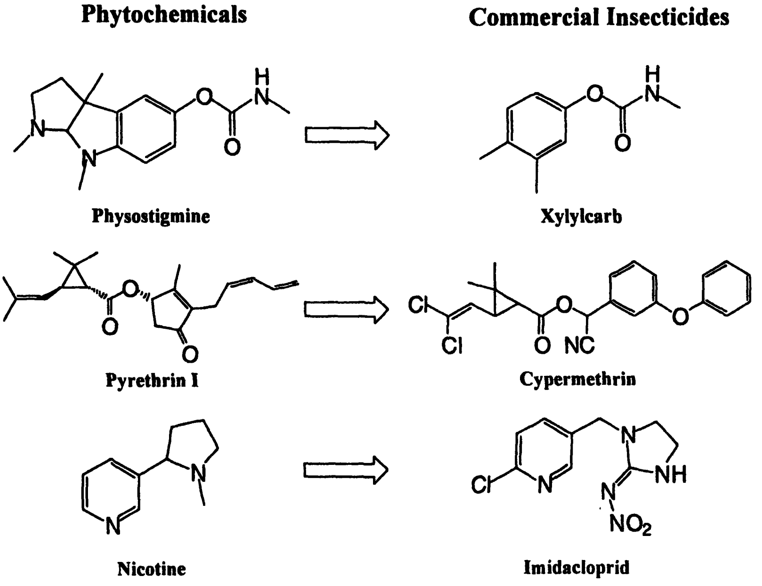


Figure 1. Insecticidal phytochemicals and commercial insecticides.

Some natural products have insecticidal activity and commercial uses in pest management. Spinosad is an insect neuro-toxin produced by soil actinomycete *Saccharopolyspora spinosa*, and is sold as a water-based suspension concentrate formulation (*7*). Rotenoid is one of the flavonoids produced by *Derris* sp. (Leguminosae) that acts as a respiration inhibitor against complex I in the electron transport system of mitochondria. The roots of *Derris* sp. is used in Asia and South America as a fish toxin. *Derris* product is marketed as a wettable powder or emulsifiable concentrate. Azadirachtin is a famous phytophagous insect antifeedant produced by the neem tree *Azadirachta indica* (Meliaceae) (*8*). This terpenoid has various biological activities, and has been incorporated in commercial products sold as an emulsifiable concentrate and in the form of solid extract (Figure 2).

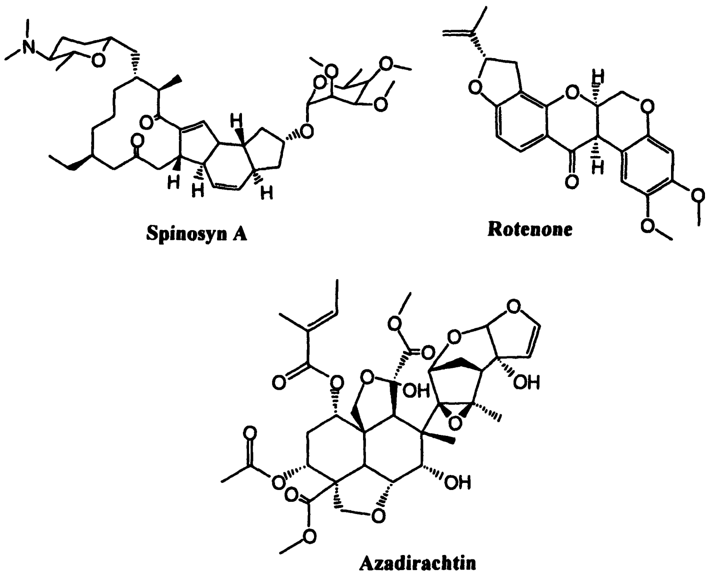


Figure 2. Natural products that have insecticidal activity.

Hexane, ether and methanol extracts of 108 species of Japanese wild plants were tested for their insect antifeedant activity against *Spodoptera litura*. Some hexane extracts of *Cyperus* spp. and *Gnapahlium affine* revealed insect antifeedant activity in this screening test.

Downloaded by STANFORD UNIV GREEN LIBR on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch014

Insect antifeedants in *Cyperus* spp.

Even when pests do damage to crops, wild Cyperaceae are not damaged in upland and paddy fields, with the notable exception of damage by the Cyperaceae specialist moth, *Calamotropha shichito* larvae. Whereas these plant species have a chemical defense system, the specialist insect seems to have overcome the plants' defense system. Therefore, specialist insect can be used as a probe to search for a plant that have defensive mechanism against this insect. Constituents of Cyperaceae extract and essential oil have been examined in studies (*9*) of allelopathy (*10*) and pharmacy, identifing many natural products, including coumarins (*11*), unique quinones (*12*) and sesquiterpenes. For example, sesquiterpene ketone, α-cyperone produced by *C. rotundus*, showed inhibition of the biosysnthesis of prostaglandine and insecticidal activity against diamondback moth (DBM) larvae (*13*). *Cyperus iria* produces the same JH III utilized as a juvenile hormone by insects (*14*). We investigated the insect antifeedant activities of extracts of Japanese and Thai Cyperaceae.

Hexane extracts made from 17 species of Japanese Cyperus and 9 species of Thai Cyperus were investigated. The most highly active extracts were *C. brevifoluis* var. *leiopis, C. flavidus, C. iria, C. nipponicus* and *C. orthostachyus* in Japanese Cyperaceae. The hexane extracts of *C. distans, C. javanicus, C. stoloniferus* showed good insect antifeedant property in this test. We isolated the active compounds remirol and cyperaquinone from *C. nipponicus* (*15*). Subsequently, we isolated large amounts of remirol and cyperaquinone from *C. eragrostis*, scabequinone from *C. distans*, cyperaquinone and dihydro-cyperaquinone from *C. tuberosus* for detailed study (Figure 3). These cyperaquinones have chemical structures of the difuranobenzoquinone type unique to the *Cyperus* genus. The dihydrobenzofuran, remirol is a precusor of these cyperaquinones (*16*), and we regarded it easier to prepare derivatives by organic synthesis for SAR study.

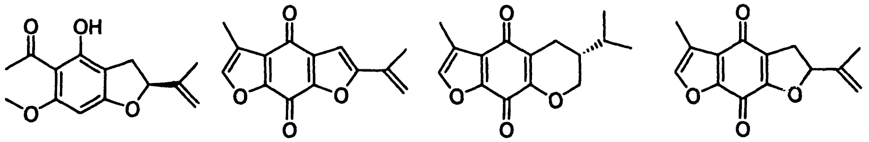
Downloaded by STANFORD UNIV GREEN LIBR on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch014

SAR of Dihydrobenzofurans

The synthesis of the 2,3-dihydrobenzofuran moiety by condensation of phenol and alkylbromide has been conducted by Yamaguchi *et al.*, (*17*). Dihydrobenzofuran derivatives with various structural differences were prepared to evaluate the effect on the feeding response of *S. litura* 3rd instar larvae. In all bioassays, the tested compounds were evaluated as racemic mixture, and the results indicated that certain substituents affected the insect antifeedant activity. With an ED_{50} value of 5.4×10^{-9} mol/cm^2, 7-acetyl-2-isopropenyl-4,6-dimethoxy-2,3-dihydrobenzofuran was among the most active insect antifeedants yet recorded. However, it is also important to find analogues with activity higher

Downloaded by STANFORD UNIV GREEN LIBR on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch014

than that of the natural product, and to elucidate the structural requirements for agents which inhibit feeding. An examination of the insect antifeedant activities of many of the benzofuransrevealed that hydroxyl derivatives had a decreased insect antifeedant activity.



(+)-Remirol Cypreaquinone (+)-Scabequinone (+)-Dihydrocyperaquinone

Figure 3. Insect antifeedants in Cyperus *spp.*

The derivatives without a substituent on the benzene ring showed weak inhibitory effect on insect feeding. However, when a methoxy group was introduced into the aromatic ring, insect feeding was markedly inhibited. Furthermore, the number of methoxy group was related to this activity. A comparison of the methoxy derivatives revealed that the most effective number of methoxy group is two or three. Substitution with an acetyl group also increased the antifeedant activity. Interestingly, 5-monoacetyl derivatives and 7-monoacetyl derivatives had completely different antifeedant activity and polarity as detected by TLC analysis. Their physicochemical properties seem to have significantly influenced their inhibitory effects on insect feeding. The importance of introducing an acetyl group at the 7-position of the benzene ring is also supported by results for other 7-acetyl derivatives. On the other hand, the introduction of a hydrophilic group, *e.g.* a hydroxyethyl derivative tended to decrease the insect antifeedant activity (*18*).

There appeared to be a relationship between the lipophilicity and antifeedant activity of the test compounds. In this study, we adopted the R_f value obtained from TLC analysis with the same solvent, because the compounds had similar chemical structures and polarity. The most effective compounds in the antifeedant bioassay showed high polarity (a small R_f value) by TLC analysis (Figure 4) (*18*). This suggested that the compounds required a certain degree of lipophilicity in order to be transported to the receptor site or target organ. In general, even if an agent has potential activity, it will be ineffective when it is unable to penetrate biological membranes. Additionally, their hydrogen bonding property is important for the appearance of this biological activity.

Based on the differences in inhibitory activity of 2,3-dihydrobenzofuran toward insect feeding, we conclude that the introduction of an acetyl or methoxy group on the aromatic ring produced effective insect antifeedant activity; on the contrary, conversion to a hydroxyl group decreased the biological activity. Moreover, the position of the substituted acetyl group appreciably affected the activity. There is a tendency for the Rf values of the test compounds to be related to their activity. Though, these dihydrobenzofurans did not show phytotoxicity, these compounds should be helpful as lead compouds for the development of agrochemicals.

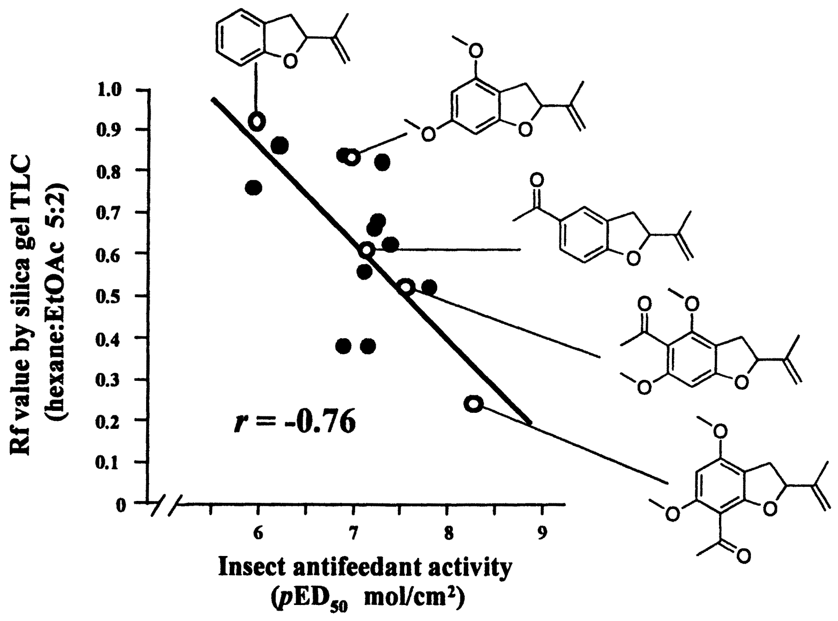


Figure 4. Correlation of the insect antifeedant activity and for the dihydrobenzofuran derivatives tested in this study. (Reproduced from reference 18. Copyright 1999.)

Simple benzoquinone from *Cyperus* sp.

The natural quinone, hydroxydietrichequinone (3-heptadec-8-enyl-2-hydroxy-5-methoxy-[1,4]benzoquinone) was produced by tropical Cyperacea, *C. javanicus*. This species showed good insect antifeedant activity in the screening of Thai *Cyperus* hexane extracts. This quinone inhibited both mitochondrial respiration and photosynthesis in their electron transport systems. The quinone had a mode of action against the ubiquinone reductase site from the results of different electron donor experiments on intact mitochondria from rat liver. The electron transport system of chloroplast from spinach leaves was inhibited by the simple benzoquinone. This benzoquinone has a C17 aliphatic chain with a double bond at its midpoint, thus 8-9 unsaturated, as determined by MS analysis of the methylthio-adduct, and spectral data indicated the configuration in a *cis*

Downloaded by STANFORD UNIV GREEN LIBR on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch014

form (Figure 5) (*19*). This biological activity and chemical structure are similar to an allelochemical, sorgoleone in *Sorghum bicolor* (*20*).

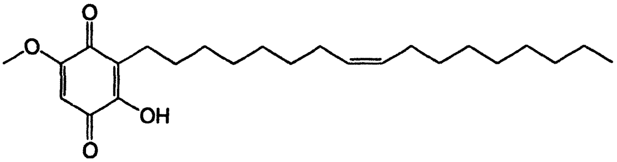
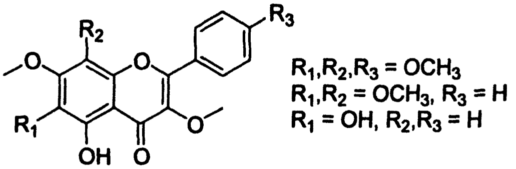


Figure 5. Blue pigment, hydroxydietrichequinone from C. javanicus.

Downloaded by STANFORD UNIV GREEN LIBR on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch014

Isolation of Insect Antifeedants from *G. affine*

In the first screening of extracts from Japanese wild plants, both the hexane and ether extracts of *G. affine* showed potential insect antifeedant activities. TLC autography bioassay of these extracts suggested some active compounds, which were isolated by column chromatography and other procedures. Based on spectral analyses, the antifeedants isolated were identified as polymethylated tree flavonoids, 5-hydroxy-3,6,7,8-tetramethoxyflavone, 5,6-dihydroxy-3,7,-dimethoxyflavone and 5-hydroxy-3,6,7,8,4'-pentamethoxyflavone (Figure 6) (*21*). The 5-hydroxy-3,6,7,8-tetra-methoxyflavone and 5,6-dihydroxy-3,7,-dimethoxyflavone were shown to have the strongest insect antifeedant activities in this study, while 5-hydroxy-3,6,7,8,4'-pentamethoxyflavone was found to have less activity than the previous two flavonoids. From a chemical ecology point of view, this plant has a woolly surface, and this feature acts as a physical defense. Even specialist moth larvae can not feed on this woolly surface (Figure 7). These insect antifeedant polymethylated flavonoids were not detected in other *Gnaphalium* sp. in Japan that have more woolly surfaces than *G. affine*, as observed under SEM, *i.e.*, *G. pennsylvanicum*, *G. spicatum* and *G. purpureum* (Figure 8). These facts suggested that *G. affine* may have evolved a chemical defense system for protection from phytophagous insects instead of a physical defense system like other *Gnaphalium* spp.



$R_1,R_2,R_3 = OCH_3$
$R_1,R_2 = OCH_3, R_3 = H$
$R_1 = OH, R_2,R_3 = H$

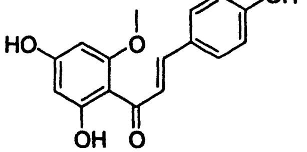


Figure 6. Insect antifeedant flavonoids in G. affine

Downloaded by STANFORD UNIV GREEN LIBR on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch014

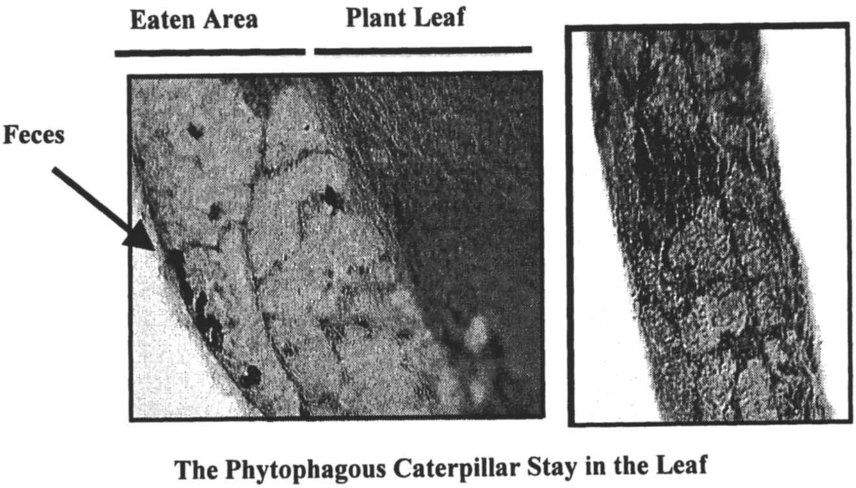

Eaten Area
Plant Leaf
Feces
The Phytophagous Caterpillar Stay in the Leaf

Figure 7. G. affine *leaf. Specialist feeding can not feed on its woolly surface.*

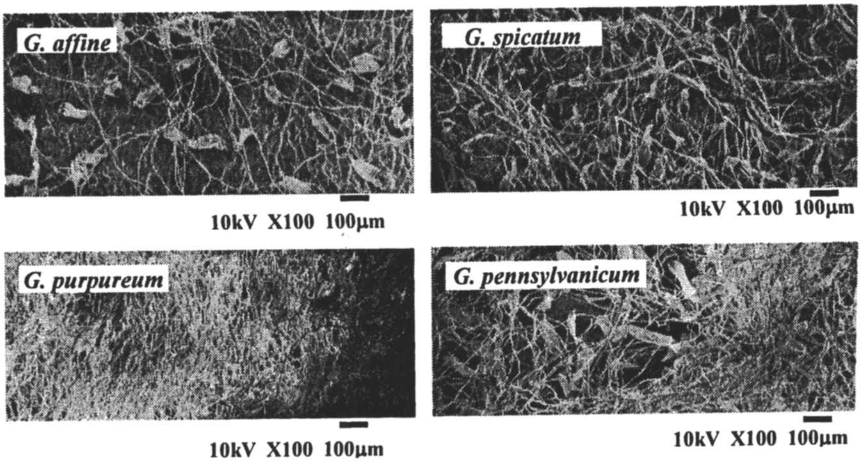

G. affine
10kV X100 100µm
G. spicatum
10kV X100 100µm
G. purpureum
10kV X100 100µm
G. pennsylvanicum
10kV X100 100µm

Figure 8. SEM of woolly filaments on the leaf surface of Gnaphalium *spp.*

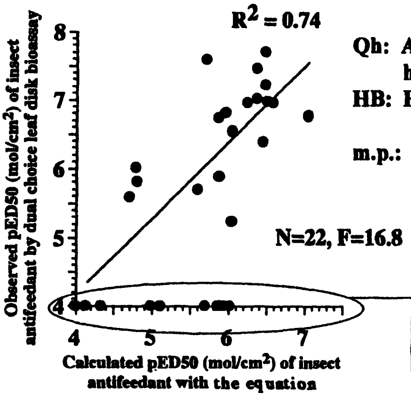
We have evaluated the insect antifeedant activity of other methylated flavonoids. Quercetin is widespread in plants and acts as a defense compound against some phytophagous insects. However, quercetin and its methylated derivatives did not show insect antifeedant activity against *S. litura* larvae. This fact suggested that not all of the methylated flavonoids have insect antifeedant activity against *S. litura* larvae (*21*).

These biologically active flavonoids were polymethylated, excluding the phenol having hydrogen bonding with the carbonyl group at the 5-position of the flavones. Remarkably, highly active flavones are those with a simple phenyl group as a B ring. Based on the bioassay evaluation, introduction of a methylether on the B-ring of the flavonoid decreases the insect antifeedant activity. This hypothesis is supported by comparison of the test compounds, the methylated quercetin and methylated flavonoids in *G. affine* which have a number of diverse substituents on the B-ring. Similarly, a methylated chalcone was isolated from the ether extract of *G. affine*. In comparison, the chalcone had a weaker activity than the three flavonoids in *G. affine*, but the amount of chalcone in the plant was larger than these flavonoids. This fact suggested that both the chalcone and the flavonoids act as defense compounds for the plant against phytophagous insects. A comparison of the insect antifeedant activity of flavones and chalcones showed that the important structural feature was the ether linkage system consisting of the pyran ring (*21*).

Downloaded by STANFORD UNIV GREEN LIBR on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch014

QSAR of Benzopyranone for Insect Antifeedant Activity

In the preceeding paragraph, we suggested that absence of substituents on the B-ring of the flavonoid conferred beneficial antifeedant activity against *S. litura* larvae. Flavonoids with a phenyl group on the B-ring and chromones, products of the elimination of the B-ring from flavonoids, were used to test this hypothesis. Even though they were 2-phenyl flavonoids, some of the test compounds did not show any antifeedant activity against *S. itura* larvae, and these inactive flavonoids also lacked a substituent at the 6-position on the A-ring. Furthermore, 6-substituted derivatives showed strong antifeedant activity against the common cutworm. Moreover, flavonoids with a hydroxyl group as a substituent at any position tended to increase this biological activity. These facts suggest that the substituent at the 6-position strongly affects the flavonoid antifeedant activity while hydrophilic substituents decreased this activity. Baicalein (5,6,7-trihydroxy flavone) derivatives did not show any insect antifeedant activity, even though this is a 6-substituent derivative. Although some chromones showed increased activity a bulky B-ring was detrimental to antifeedant activity (*22*). QSAR analysis of all test benzopyranones showed that one of the most important physicochemical property related to insect antifeedant activity in this test is melting point (Figure 9). The fact that the efficacy of some pesticides is affected by their melting point was known (*23*). Our analysis allows elimination of compounds with high melting points from the set for QSAR analysis. Our results suggest that charges on C(3) and C(5) of the flavonoid are important for this biological activity. In addition, an adequate hydrogen bonding property, which is different from lipophilicity, contributes to this activity based on a QSAR analysis (Figure 10)(*22*).

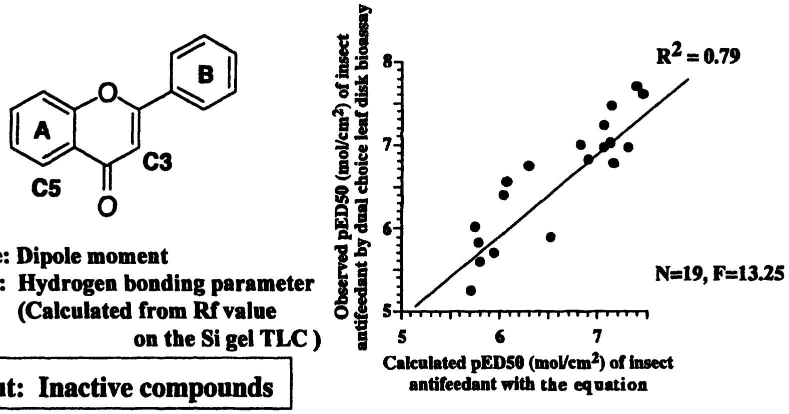


Qh: Atomic charge on the most positively charged hydrogen atom in the molecule
HB: Hydrogen bonding parameter (Calculated from Rf value on the Si gel TLC)
m.p.: Melting point of the compounds

Almost inactive compounds have high melting point

$$pED_{50} = 1.247\ (\pm 2.29)\ Qh + 1.25\ (\pm 0.87)\ HB - 0.01\ (\pm 0.003)\ mp + 7.925$$

Figure 9. Adapation of classical QSAR analysis for insect antifeedant activity of benzopyranones (flavonoids). (Reproduced from reference 22. Copyright 2003 American Chemical Society.)



dipole: Dipole moment
HB : Hydrogen bonding parameter (Calculated from Rf value on the Si gel TLC)

Out: Inactive compounds

$$pED_{50} = -0.129\ (\pm 0.13)\ dipole + 6.434\ (\pm 1.56)\ \delta C3 - 5.29\ (\pm 1.12)\ \delta C5$$

Figure 10. Re-analysis of QSAR for insect antifeedant activity of benzopyranones (flavonoids) eliminated inactive compound from training sets. (Reproduced from reference 22. Copyright 2003 American Chemical Society.)

Downloaded by STANFORD UNIV GREEN LIBR on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch014

Conclusion

Biological activity-guided isolation has led to the identification of quinones and dihydrobenzofuran as insect antifeedants in *Cyperus* spp., and methylated flavonoids in *G. affine*. Chemical structure activity relationship studies were conducted to determine the essential structural requirements for insect antifeedant activity of these natural products. It was shown that the insect antifeedants have a more simple chemical structure than recently reported natural products. These data provide a simpler approach to developing an insect control agent. Although these compounds did not kill the target insect, it is not always desirable to have high efficacy in agro-environmental conditions. Introduction of an acetyl group and/ or methoxyl group on the benzene ring to decrease the melting point may help in the development of new agrochemicals. There are a few flavonoid-type pesticides, but we know that flavonoids have diverse functions in chemical ecology. Phytophenolics could be used to protect crops from insects.

Downloaded by STANFORD UNIV GREEN LIBR on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch014

References

1. Morimoto, M.; Komai, K. *Recent Res. Devel. Phytochem.* **2004**, 99-114.
2. *Anti-feeding-Compounds;* Norris, D. M.; Haug G.; Hoffmann, H., Eds.; Springer-Verlag: Berlin, 1981; pp. 99-144.
3. Miyakado, M.; Watanabe, K.; Miyamaoto, J. In *Phytochemicals for Pest Control*; Hedrin, P. A.; Hollingworth, R. M.; Masler, E. P.; Miyamoto, J. Thompson, D. G., Eds; ACS Symp. Ser. 658; American Chemical Society: Washington DC, 1997; pp. 168-182.
4. *Carbamate Insecticides: Chemistry Biochemistry and Toxicology*; Kuhr, R. J.; Dorough, H. W., Eds. CRC Press: Cleveland, OH, 1976; p 301.
5. Katsuda, Y. *Pestic. Sci.* **1999,** *55*, 775-782.
6. Shiokawa,K.;Moriya, K.; Shibuya, K.; Hattori, Y.; Tsuboi, S.; Kagabu, S. *Biosci. Biotechnol. Biochem.* **1992,** *56*, 1364.
7. Thompson, G. D.; Dutton, R.; Sparks, T. C. *Pest Manag. Sci.* **2000,** *56*, 696-702.
8. Mordue, L. A. J.; Blackwell, A. *J. Insect Physiol.* **1993,** *30*, 903-924.
9. Sonwa, M. M.; König, W. A. *Phytochemistry* **2001,** *58*, 799-810.
10. Komai, K.; Tang, C. S.; Nishimoto, R. K. *J. Chem. Ecol.* **1991,** *17*, 1-8.
11. Dini, A.; Ramundo, E.; Saturnino, P.; Scimone, A.; Stagno d'Alcontres, I. *Biochem. Syst. Ecol.* **1993,** *21*, 305.
12. Allan, R. D.; Wells, R. J.; Correll, R. L.; MacLeod, J. K. *Phytochemistry* **1978,** *17*, 263-266.
13. Dadang; O., K.; Kato, S.; Yamamoto, I. *J. Pestic. Sci.* **1996,** *21*, 444-446.
14. Toong, Y. C.; Schooley, D. A.; Baker, F. C. *Nature* **1988,** *333*, 170-171.

15. Morimoto, M.; Fujii, Y.; Komai, K. *Phytochemistry* **1999**, *51*, 605-608.
16. Allan, R. D.; Correll, R. L.; Wells, R. J. *Tetrahedron Lett.* **1969**, 4669-4672.
17. Yamaguchi, S.; Takai, M.; Hanazome, I.; Okada, Y.; Kawase, Y. *Bull. Chem. Soc. Jpn.* **1987**, *60*, 3603-3605.
18. Morimoto, M.; Urakawa, M.; Fujitaka, T.; Komai, K. *Biosci. Biotechnol. Biochem* **1999**, *63*, 840-846.
19. Morimoto, M.; Shimomura, Y.; Mizuno, R.; Komai, K. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 1849-1851.
20. Rimando, A. M.; Dayan, F. E.; Czarnota, M. A.; Weston, L. A.; Duke, S. O. *J. Nat. Prod.* **1998**, *61*, 927-930.
21. Morimoto, M.; Kumeda, S.; Komai, K. *J. Agric. Food Chem.* **2000**, *48*, 1888-1891.
22. Morimoto, M.; Tanimoto, K.; Nakano, S.; Ozaki, Y.; Nakano, A.; Komai, K. *J. Agric. Food Chem.* **2003**, 51, 389-393.
23. Baker, E. A.; Hayes, A. L.; Butler, R. C. *Pestic. Sci.* **1992**, *34*, 167-182.

Downloaded by STANFORD UNIV GREEN LIBR on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch014

Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch015

Chapter 15

Isolation and Identification of an Insecticidal Soyasaponin from Field Pea Extracts

Wesley G. Taylor[1] and Paul G. Fields[2]

[1]Saskatoon Research Centre, Agriculture and Agri-Food Canada,
107 Science Place, Saskatoon, Saskatchewan S7N 0X2, Canada
[2]Cereal Research Centre, Agriculture and Agri-Food Canada,
195 Dafoe Road, Winnipeg, Manitoba R3T 2M9, Canada

Dehydrosoyasaponin I was identified as a minor component with antifeedant and insecticidal properties in extracts from yellow field peas (*Pisum sativum* L.). Chromatographic fractionation of crude methanolic extracts (C8 extracts) from commercial protein-rich pea flour yielded fractions containing soyasaponins and phospholipids in addition to other fractions containing the insecticidal pea albumin 1b family of cysteine-rich plant peptides. Activity of the saponin fractions was determined by a flour disk bioassay with the rice weevil [*Sitophilus oryzae* (L.)], an insect pest of stored products. Synergists of dehydrosoyasaponin I were also present in C8 extracts, in the form of three phospholipids of the lysolecithin (lyso-phosphatidylcholine) type.

In Natural Products for Pest Management; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

Certain legume plants, including seeds of the pea (*Pisum sativum* L.), are toxic to insects (*1-4*). Bodnaryk et al. (*5*) showed that commercial flour from field peas was insecticidal to stored-product insects. Protein-rich pea flour was more effective against stored-product insects than starch- or fibre-rich fractions (*5, 6*). An extraction procedure was developed with hot 80% methanol (20% water) to obtain crude insecticidal pea extracts. The aqueous methanol extracts from defatted, protein-rich flour were partially purified with reversed phase C8 silica (*5*). Activity was found in fractions obtained by elution of the C8 silica column with 100% methanol. These C8 extracts displayed antifeedant and insecticidal activity against rice weevil [*Sitophilus oryzae* (L.)] and other stored-product insects but the active ingredients of C8 extracts were not identified.

In granary trials (*7*), protein-rich pea flour at a concentration of 0.1% reduced *S. oryzae* by 90% and rusty grain beetle [*Cryptolestes ferrugineus* (Stephens)] and red flour beetle [*Tribolium castaneum* (Herbst)] by 70%. However, this concentration was probably too high for practical use as a grain protectant. Insect pests infesting stored grains are primarily controlled by organophosphorus insecticides, ozone-depleting methyl bromide and toxic phosphine gas (*8*). Alternate approaches for insect control with safe and effective natural products are needed, especially from food-grade materials.

The purpose of our research was to isolate and identify the anti-insect components of pea extracts derived from air-classified protein-rich field pea flour. This chapter describes bioassay-guided fractionation by silica gel chromatography of C8 extracts, characterizing active components contained in fractions of intermediate polarity (*9*). Highly polar end fractions from C8 extracts were found (*10, 11*) to contain mixtures of cysteine-rich peptides of the pea albumin 1b (PA1b) type (*12*). Some of these PA1b peptides had previously been extracted from peas with acetate buffer and were shown to be effective against stored-product insect pests (*13*).

Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch015

Experimental C8 Extracts and Insect Bioassays

Protein-rich pea flour, obtained by an air-classification process (*14*), was supplied by Parrheim Foods Limited (Saskatoon, Canada). The flour was extracted in the laboratory with 80% methanol (*5*) (Figure 1). Utilizing two C8 SepPak Vac™ cartridges per 100 g of flour, the C8 extracts in methanol were combined and concentrated to dryness with a centrifugal Savant evaporator. Starting with 100 grams of defatted protein-rich flour, a beige C8 powder was obtained in 0.7-0.9% yield.

Antifeedant activity was assessed with a flour disk bioassay (*15*) with 70% ethanol as solvent. Twenty five adult *S. oryzae* (1-2 weeks old) were held on five wheat flour disks for three days at 30 °C, 70% relative humidity. Flour disks (ca. 0.1 g/disk) were weighed before and after exposure to the insects. Antifeedant activity was determined by expressing consumption of treated disks

Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch015

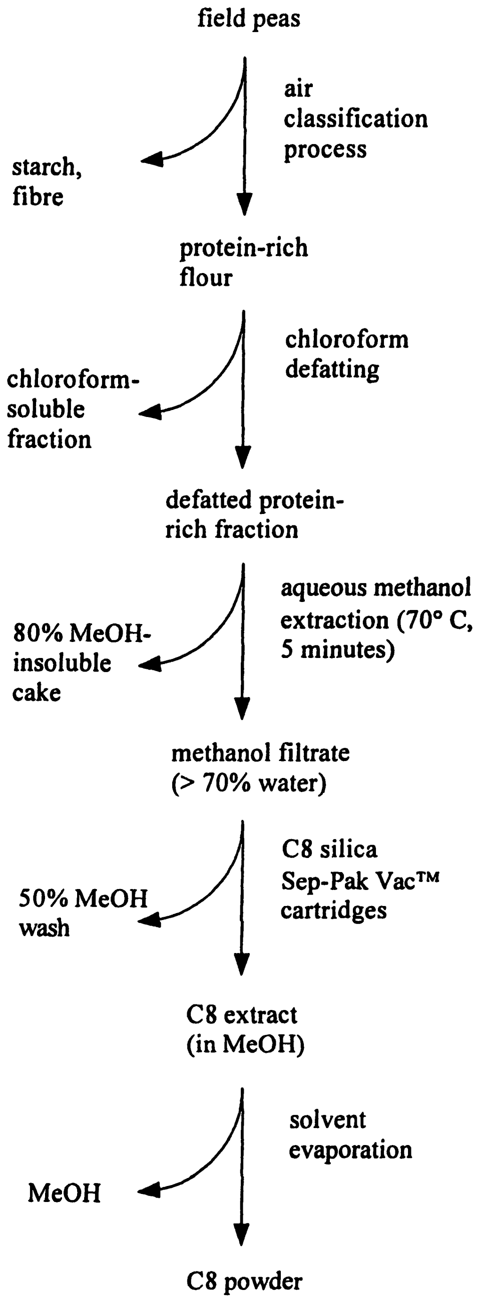

field peas
air classification process
starch, fibre
protein-rich flour
chloroform defatting
chloroform-soluble fraction
defatted protein-rich fraction
aqueous methanol extraction (70° C, 5 minutes)
80% MeOH-insoluble cake
methanol filtrate (> 70% water)
C8 silica Sep-Pak Vac™ cartridges
50% MeOH wash
C8 extract (in MeOH)
solvent evaporation
MeOH
C8 powder

Figure 1. Processing steps employed during isolation of insecticidal C8 extracts.

as a percentage of control disks (70% ethanol). Positive controls using the same C8 extract were run with each bioassay. Insecticidal activity was assessed as median survival time by Kaplan-Meier survival analysis (log-rank) using SigmaStat (SPSS Inc., Chicago, IL). After weighing the disks, *S. oryzae* and flour disks were returned to the Petri dishes and survivors noted each day until the insects had been on the disks for a total of 14 days. A close correlation was previously found between antifeedant activity and toxicity of pea extracts (*5, 16*).

Fractionation of C8 Powder

Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch015

Initially, samples of C8 extracts were separated by column chromatography with silica gel and chloroform-methanol mixtures. The C8 powder gave two chromatographically distinct, medium to high polarity bands that showed good antifeedant activity. The first active band, appearing in the 40% chloroform (60% methanol) fraction and designated C8-1a, represented about 10% of the mass of applied C8. It reduced food consumption to 15% of untreated samples. The second active, highly polar band eluted gradually with 80-100% methanol and was collected as five separate fractions at the end of the experiment. These end fractions, collectively representing about 25% of the applied C8, gave food consumption values of 24-45%. The most active of these five fractions, designated as C8-1b, was compared in a dose-response experiment to C8-1a and to impure C8 (Figure 2).

Samples of C8, C8-1a and C8-1b, each with practically identical antifeedant activity, were spotted on analytical TLC plates (EM Science plastic sheets, 0.2 mm layer thickness) and developed with a mixture designated as solvent system 1 (the lower layer of chloroform-methanol-water: 65-35-10, by volume). By employing ultraviolet light plus TLC detection reagents (applied as sprays to the developed plates and exemplified by ninhydrin and Liebermann-Burchard reagents), it could be demonstrated that C8, C8-1a and C8-1b were complex mixtures. However, certain of the TLC spots in the sample of C8-1a gave a grey colored response to Liebermann-Burchard, indicative of the presence of triterpene saponins such as soyasaponin I (*17*) whereas most of the spots from C8-1b were positive to ninhydrin, indicating that this fraction probably contained peptides (*18*). This implied that at least two chemically distinct insecticidal components were present in the C8 extract.

A technique was sought that was faster than column chromatography to fractionate the C8 mixture into the equivalent of C8-1a and C8-1b, ensuring that there was a clear separation between these insect-active fractions. This objective was achieved by flash chromatography using a FLASH 40 M^{TM} apparatus (Biotage Inc., Charlottesville, VA) equipped with a prepacked 90 gram (4 x 15

Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch015

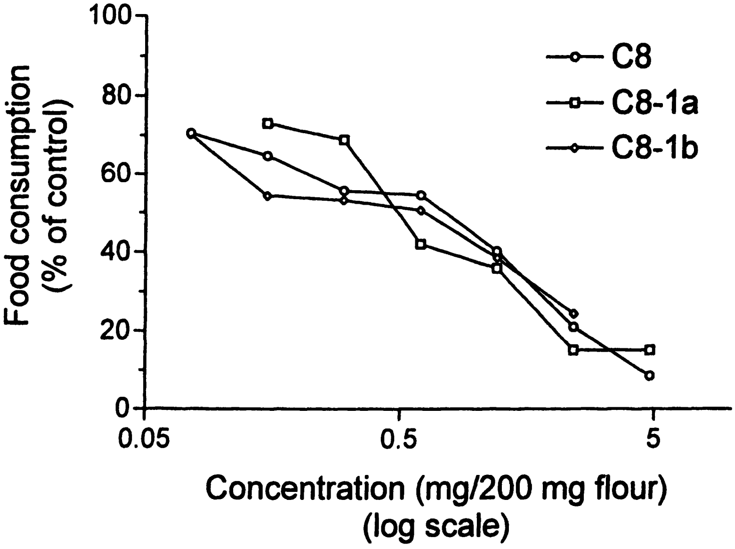


Figure 2. Comparison of dose-response curves in antifeedant bioassays with S. oryzae using a crude C8 extract and partially purified C8-1a and C8-1b extracts. (Reproduced from reference 9. Copyright 2004 American Chemical Society.)

cm) KP-Sil^{TM} (Biotage) cartridge (32-63 :m, 60 Δ silica). Flow rate was maintained at 20 ml/min with solvent system 1. The properties of the collected fractions are shown (Table I).

Table I. Fractionation of a C8 Extract (250 mg) by Flash Chromatography with a Silica Cartridge.

Fraction	R_F [a]	*Yield (mg)*	*F.C. (%)* [b]
1	>0.5	65	109
2 (C8-2a)	0.25-0.5	48	39
3	<0.25	7	71
4 (C8-2b) [c]	<0.25	28	52

[a] With silica gel TLC plates.

[b] Food consumption, expressed as % of control. Experimental samples were tested in the *S. oryzae* antifeedant bioassay at a concentration of 1.6 mg/200 mg flour (1.2 mg for fraction 3).

[c] This end fraction was obtained by elution with methanol.

Source: Reproduced from reference 9. Copyright 2004 American Chemical Society.

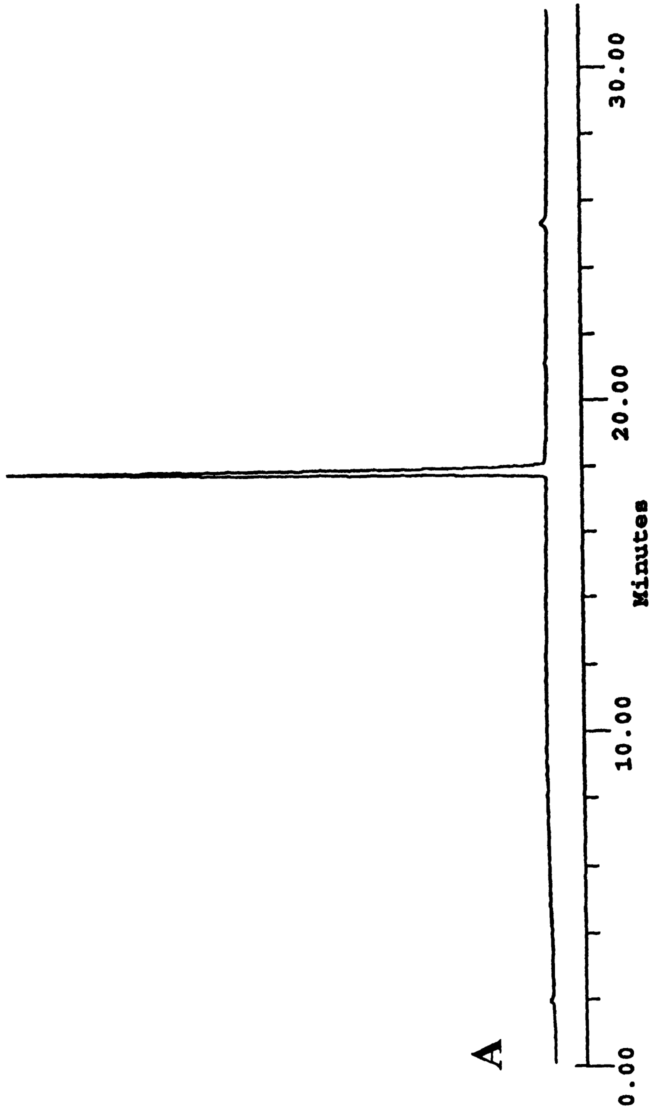
Flash chromatography with silica cartridges was effective at separating nonpolar inactive material (fraction 1) in C8 samples. No antifeedant activity could be demonstrated in this major fraction. Fraction 2, designated C8-2a and representing about 20% of the mass of applied C8, was similar in activity and TLC profile (Liebermann-Burchard positive spots) to C8-1a from the silica gel column. Fraction 4, designated as C8-2b, represented an 11% yield of high polarity, ninhydrin-positive components that were similar to C8-1b from the column. Fraction C8-2b was shown to contain peptides of the PA1b type (*10*).

Identification of Components

Evidence from TLC and HPLC indicated that soyasaponin I (S-I) was a major component in extracts of C8-2a. Thus, authentic samples of S-I showed the same TLC properties as the major component of C8-2a, using various solvent systems and spray reagents. HPLC clearly illustrated the similarity of these two samples, as illustrated by the ELSD traces shown in Figure 3. Although several late eluting minor components were found in C8-2a, it was reasonable to suspect that S-I might be responsible for the antifeedant properties of the C8-2a extract. However, Bodnaryk et al. (*5*) showed previously that their sample of S-I was nearly inactive in the rice weevil bioassays. In the present work, reference samples of S-I of various purities were isolated from pea flour and soybean meal

Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch015

Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch015



Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch015

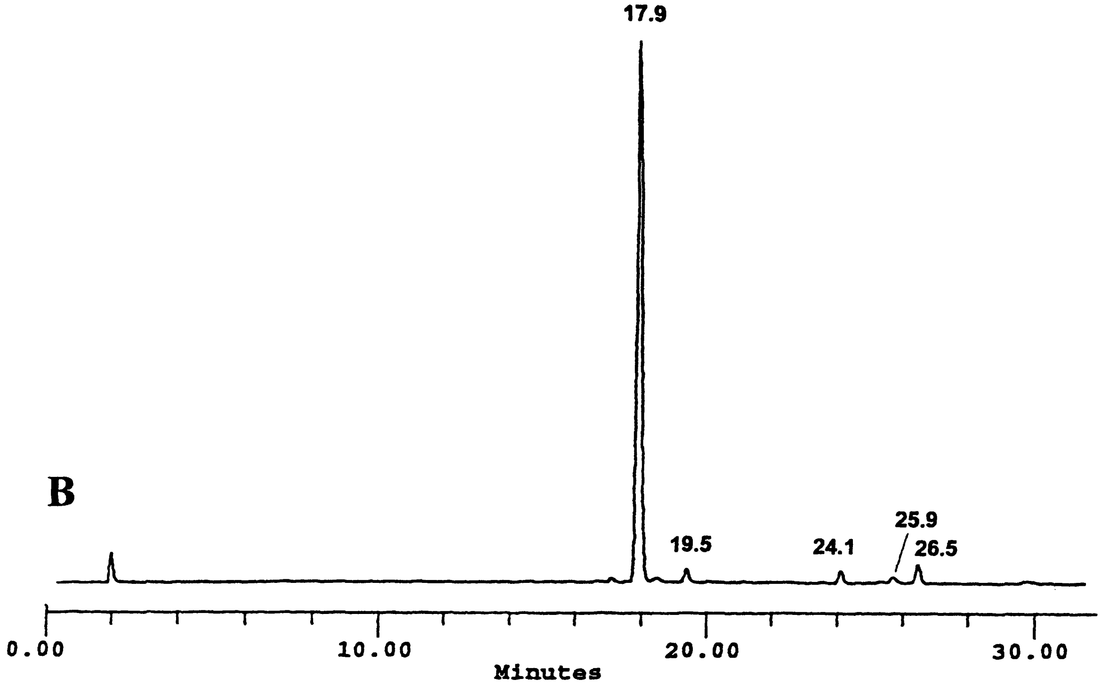


Figure 3. Comparison by HPLC with a reversed phase C18 Symmetry™ column and an ELSD of (A) a reference sample of inactive S-I and (B) an active C8-2a extract. (Reproduced from reference 9. Copyright 2004 American Chemical Society.)

(*19*, *20*). None of the preparations of S-I approached the antifeedant activity of C8-2a.

Tsurumi et al. (*21*) reported that S-I does not occur in the free form in peas but is formed during extraction from soyasaponin VI (S-VI), also known as soyasaponin ∃g, soyasaponin BeA or chromosaponin I. A mixture containing S-VI was isolated (*21*) and purified by silica gel flash chromatography. The availability of this reference sample confirmed that S-VI was present as a minor component in C8-2a, at 19.5 min (see Figure 3). It therefore seemed unlikely that S-VI, of weak antifeedant activity, could account for the moderate to high activity of C8-2a, which contained S-VI as a minor component.

Some of the minor components of C8-2a were identified to attempt to explain the antifeedant activity. This was achieved primarily by HPLC and HPLC/MS (Table II). The derived chemical structures are shown in Figure 4.

Table II. HPLC and Mass Spectral Data on Identified Components of Active Fraction C8-2a

Retention time (min)	*Relative peak areas [a] (%)*	*Quasimolecular ion [b]*	*Structure in Figure 4*
17.9	93.2	943	S-I
19.5	1.7	941	D-I
19.5	[c]	1069	S-VI
24.1	1.6	520	L-18:2
25.9	1.0	496	L-16:0
26.5	2.5	522	L-18:1

[a] Obtained by integration of HPLC peaks from a representative ELSD chromatogram (see Figure 3B) by (peak area of indicated component/sum of peak areas x 100).

[b] These are *m/z* values for protonated molecular ions found in the corresponding peaks of the total ion chromatogram during electrospray HPLC/MS analysis.

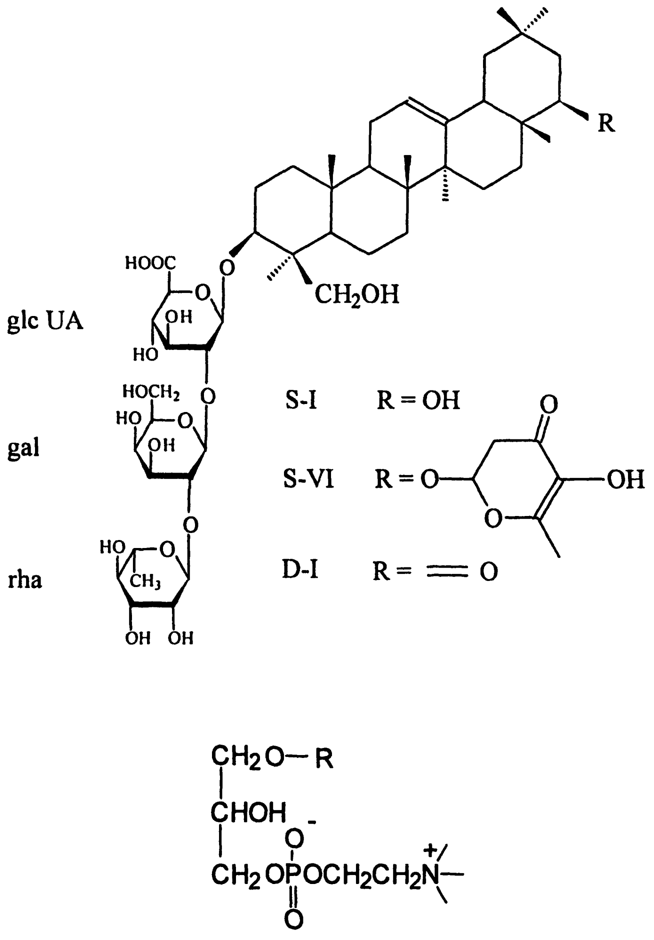
[c] Compounds D-I and S-VI coeluted during conditions used for HPLC so the area of these two components represented 1.7% of the mixture. During HPLC/MS, the leading edge of this peak showed predominantly the ion at *m/z* 941 whereas the trailing edge showed predominantly the *m/z* 1069 ion.

Source: Reproduced from reference 9. Copyright 2004 American Chemical Society.

The major HPLC peak at 17.9 min in C8-2a was confirmed as S-I by electrospray HPLC/MS. The peak at 19.5 min was of particular interest because not only the quasimolecular ion for S-VI was observed but also another prominent ion at m/z 941 that corresponded to the molecular weight of dehydrosoyasaponin I (D-I), a known saponin (*22*, *23*).

Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch015

Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch015


R
CH2OH
HOOC
O
glc UA
OH
HO
HOCH2
gal
rha
CH3
OH OH
S-I R = OH
S-VI R = O
O
OH
D-I R = O
CH2O—R
CHOH
O-
CH2OPOCH2CH2N
+
O

L-16:0 R = $C_{16}H_{31}O$ (palmitoyl)

L-18:1 R = $C_{18}H_{33}O$ (oleoyl)

L-18:2 R = $C_{18}H_{31}O$ (linoleoyl)

Figure 4. Structures of the soyasaponins and lysolecithins identified in C8 extracts.

HPLC/MS showed that the last eluting peaks, representing about 5% of the mixture, were phospholipids of the lysolecithin (lyso-phosphatidylcholine) type (*24*). Their identity was easily confirmed because reference samples containing these compounds were available commercially. The three reference lysolecithins L-16:0, L-18:1 and L-18:2, purchased from Sigma-Aldrich, were inactive in antifeedant bioassays.

Additional Fractionation of C8-2a.

Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch015

The C8-2a extract was further purified with MCI gel CHP20P, a polymeric adsorbent resin frequently employed in saponin separation chemistry. With a flow rate of 3 ml/min (2 bed volumes/hour), the column was eluted with a step gradient of 100% water to 90% methanol in 10% increments (50 ml fractions). Elution was continued with 100% methanol. Rotary and Savant evaporation of the solvent showed that the first eluting material was contained in the 80% methanol fraction. Appropriate fractions were combined on the basis of similar TLC profiles with solvent system 1 and Liebermann-Burchard (or naphthoresorcinol) sprays. The major component of fractions 1 and 2 was S-I. Both fractions were nearly inactive. Fraction 3, representing the main fraction from the column, contained not only S-I as the major component but also D-I as a minor component. This fraction was active (35% food consumption). Fraction 4 appeared to be free of both S-I and S-VI but enriched in D-I (45% food consumption). This evidence suggested that D-I was the insecticidal factor in the C8-2a extracts, although the major components in fraction 4 were L-16:0, L-18:1 and L-18:2.

D-I was known to occur as a minor component in alfalfa (*22*), immature green peas (*23*), soybeans (*25*) and other legumes (*26-30*). In mammalian *in vitro* experiments (*30*, *31*), D-I was shown to be a high-affinity activator of calcium-dependent potassium channels and was 60 fold more potent than S-I as a potassium channel opener. To our knowledge, the influence of D-I on insects had not been reported previously.

Isolation of Dehydrosoyasaponin I (D-I).

Although MCI gel CHP20P provided reasonable separation of S-I and D-I, the latter compound could not be isolated in pure form because of the coeluting lysolecithins. HPLC with C-18 reverse phase columns did not offer a logical solution because D-I and S-VI coeluted under acidic conditions. Using RPC, we achieved good separation of all of these components with a column of polystyrene/divinylbenzene beads operated at high pH (pH 10.5) and a gradient composed of dilute ammonium hydroxide and acetonitrile. D-I was isolated in low milligram quantities by injecting saponin-rich samples from MCI gel chromatography. Small amounts of D-I could also be isolated by RPC on saponin-rich C8-2a extracts but of reduced purity. It was also possible to isolate additional small samples of S-I and S-VI by RPC.

The identity of isolated D-I was readily established by CID experiments. These experiments, summarized in Table III, were initially done on the protonated form of S-I because the daughter ion fragments of this saponin have been assigned previously (*32*). It was found that D-I fragmented under appropriate CID conditions in an entirely analogous manner, yielding the same daughter ions as S-I but 2 mass units lower, corresponding to the difference in molecular masses of the aglycones.

Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch015

Table III. Prominent Daughter Ions Observed during Collision-Induced Dissociation Experiments. [a]

Sample	*MH^+*	*$(M\text{-}rha{+}H)^+$*	*$(M\text{-}rha\text{-}gal{+}H)^+$*	*$(M\text{-}rha\text{-}gal\text{-}H_2O{+}H)^+$*	*$(M\text{-}rha\text{-}gal\text{-}2H_2O{+}H)^+$*	*(Agly.[b] $+H)^+$*	*$(Agly.\text{-}OH)^+$*	*$(Agly.\text{-}OH\text{-}H_2O)^+$*
S-I [c]	943 (100)	797 (35)	635 (20)	617 (15)	599 (20)	459 (5)	441 (55)	423 (30)
D-I	941 (100)	795 (25)	633 (15)	615 (5)	597 (15)	457 (5)	439 (40)	421 (10)

[a] The collision energy was set at 30 eV. Numbers shown are *m/z* values, with the relative intensities of the ions in brackets (Micromass Quattro LC).

[b] The aglycone of S-I is soyasapogenol B (molecular weight of 458). The aglycone of D-I is soyasapogenol E (molecular weight of 456).

[c] The indicated fragmentation pathways for S-I have been assigned (*32*).

RPC isolates of S-I, S-VI and D-I gave food consumption values in the rice weevil bioassay of 92% (1.6 mg dose), 80% (1.6 mg) and 17% (1.7 mg) respectively. Saponins have previously been suggested as possible factors in prevention of insect attacks to legume seeds (*33, 34*). Our findings implicate D-I as the insect-active soyasaponin of pea seeds.

Synergy Tests

Although the activity of D-I exceeded the activity of S-VI (and S-I), it did not seem reasonable that the presence of the low concentrations of D-I could completely account for the antifeedant effect of C8-2a extracts or of fractions of C8-2a obtained by MCI gel chromatography. Additionally, the S-VI isolated by RPC showed weaker antifeedant activity than the isolate of S-VI obtained by silica flash chromatography. This observation could be rationalized from HPLC/MS analyses because the latter isolate was contaminated not only with D-I but also with the lysolecithins.

It should be noted that samples of C8-2a, including the MCI gel purified fractions of C8-2a, appeared to be free of high-polarity ninhydrin-positive components, that is the TLC components of $R_F < 0.25$ (C8-2b of Table I). In addition, we were unable to detect these highly polar substances during HPLC of C8-2a, which were subsequently shown to elute near 15 min (see Figure 3).

We therefore examined the possibility that the lysolecithins were contributing to the antifeedant activity of C8-2a, by enhancing the activity of D-I. Low-dose experiments and mixing experiments were therefore performed, using available quantities of D-I. It was indeed found (Table IV) that the lysolecithins synergized the activity of D-I when tested with a mixture of L-16:0, L-18:1 and L-18:2. Without the addition of lysolecithins, D-I at a dosage of 0.4 mg/200 mg flour would be expected to produce little activity, similar to the 80% food consumption value and > 14 day survival time with the 0.5 mg dose of D-I (trial 3). With the four component mixture, the food consumption was 33% and the median survival time was 6.7 days (trial 4). The activity was not enhanced by addition of S-I either to the mixture of lysolecithins and D-I (trial 5) or to the lysolecithins only (trial 6). The lysolecithin mixture by itself (trial 7) was inactive, like S-I (trial 8) and S-VI (trial 9).

Lysolecithins, like saponins, are excellent detergents and can modify the function of membrane proteins including ion channels (*35*). The mechanism of synergism reported here and the optimal proportions of active ingredients will require further study.

Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch015

Table IV. Antifeedant and Insecticidal Activity of Pea Soyasaponins and Some Synergistic Mixtures.

	--- *Soyasaponin* --- *(mg)*			------- *Lysolecithin* ------- *(mg)*			*F.C.*[a]	*Median Survival Time*[b] *(days, ± SE)*
	D-I	*S-I*	*S-VI*	*L-18:2*	*L-16:0*	*L-18:1*		
1	1.7						17	5.8 ± 0.2 a
2	1.3						65	> 14 d
3	0.5						80	> 14 d
4	0.4			0.4	0.4	0.4	33	6.7 ± 0.3 b
5	0.4	0.7[c]		0.5	0.2	0.5	37	9.0 ± 0.5 c
6		0.4[c]		0.4	0.5	0.4	85	> 14 d
7				0.6	0.7	0.6	109	> 14 d
8		1.6[c]					77	> 14 d
9			1.6[d]				80	> 14 d

[a] Food consumption, expressed as % of control, in the *S. oryzae* bioassay. Samples were tested at the indicated amounts in 200 mg of flour.

[b] Kaplan-Meier survival analysis was used to estimate the median survival times and multiple comparisons were made with the Holm-Sidak method, $P = 0.05$. Medians followed by a different letter are significantly different. All insects in controls survived to 14 days.

[c] Purified by methods a, c, b and d of reference 9.

[d] Isolated by RPC with an AKTAExplorer 100 LC (Amersham Biosciences).

Source: Reproduced from reference 9. Copyright 2004 American Chemical Society.

Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch015

References

1. Coombs, C. W.; Billings, C. J.; Porter, J. E. *J. Stored Prod. Res.* **1977**, *13*, 53-58.
2. Bell, E. A. Toxins in seeds. In *Biochemical Aspects of Plant and Animal Coevolution*; Harborne, J. B., Ed.; Academic Press, NY, 1978; pp 143-161.
3. Holloway, G. J. *Bull. Ent. Res.* **1986**, *76*, 287-295.
4. Morris, J. B. Legume genetic resources with novel "value added" industrial and pharmaceutical use. In *Perspectives on New Crops and Uses*; Janick, J., Ed.; ASHS Press, Alexandria, VA, 1999; pp 196-201.
5. Bodnaryk, R. P.; Fields, P. G.; Xie, Y.; Fulcher, K. A. U. S. Patent 5,955,082. **1999**.

6. Fields, P. G.; Xie, Y. S.; Hou, X. *J. Stored Prod. Res.* **2001**, *37*, 359-370.
7. Hou, X.; Fields, P. G. *J. Econ. Entomol.* **2003**, *96*, 1005-1015.
8. Arthur, F. H. *J. Stored Prod. Res.* **1996**, *32*, 293-302.
9. Taylor, W. G.; Fields, P. G.; Sutherland, D. H. *J. Agric. Food Chem.* **2004**, *52*, 7484-7490.
10. Taylor, W. G.; Fields, P. G.; Elder, J. L. *J. Agric. Food Chem.* **2004**, *52*, 7491-7498.
11. Taylor, W. G.; Sutherland, D. H.; Olson, D. J. H.; Ross, A. R. S.; Fields, P. G. *J. Agric. Food Chem.* **2004**, *52*, 7499-7506.
12. Higgins, T. J. V.; Chandler, P. M.; Randall, P. J.; Spencer, D.; Beach, L. R.; Blagrove, R. J.; Kortt, A. A.; Inglis, A. S. *J. Biol. Chem.* **1986**, *261*, 11124-11130.
13. Delobel, B.; Grenier, A.; Gueguen, J.; Ferrasson, E.; Mbailao, M. PCT WO99/58695. **1999**.
14. Tyler, R. T.; Youngs, C. G.; Sosulski, F. W. *Cereal Chem.* **1981**, *58*, 144-148.
15. Xie, Y. S.; Bodnaryk, R. P.; Fields, P. G. *Can. Entomol.* **1996**, *128*, 865-875.
16. Hou, X.; Fields, P.; Taylor, W. *Can. Entomol.* **2004**, *136*, 581-590.
17. Hostettmann, K.; Marston, A. *Saponins.* Cambridge University Press, Cambridge, UK, 1995; p 124.
18. Stahl, E. *Thin Layer Chromatography. A Laboratory Handbook*, 2nd ed., Springer-Verlag, NY, 1969; p 568.
19. Kitagawa, I.; Yoshikawa, M.; Imakura, Y.; Yosioka, I. *Chem. Pharm. Bull.* **1974**, *22*, 1339-1347.
20. Kitagawa, I.; Yoshikawa, M.;Yosioka, I. *Chem. Pharm. Bull.* **1976**, *24*, 121-129.
21. Tsurumi, S.; Takagi, T.; Hashimoto, T. *Phytochemistry* **1992**, *31*, 2435-2438.
22. Kitagawa, I.; Taniyama, T.; Murakami, T.; Yoshihara, M.; Yoshikawa, M. *Yakugaku Zasshi* **1988**, *108*, 547-554.
23. Murakami, T.; Kohno, K.; Matsuda, H.; Yoshikawa, M. *Chem. Pharm. Bull.* **2001**, *49*, 73-77.
24. Khaselev, N.; Murphy, R. C. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 283-291.
25. Kudou, S.; Tonomura, M.; Tsukamoto, C.; Shimoyamada, M.; Uchida, T.; Okubo, K. *Biosci. Biotech. Biochem.* **1992**, *56*, 142-143.
26. Kubo, T.; Hamada, S.; Nohara, T.; Wang, Z.; Hirayama, H.; Ikegami, K.; Yasukawa, K.; Takido, M.. *Chem. Pharm. Bull.* **1989**, *37*, 2229-2231.
27. Konoshima, T.; Kozuka, M.; Haruna, M.; Ito, K. *J. Nat. Prod.* **1991**, *54*, 830-836.

Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch015

28. Ding, Y.; Takeshita, T.; Yokoyama, K.; Kinjo, J.; Nohara, T. *Chem. Pharm. Bull.* **1992,** *40*, 139-142.
29. Miyao, H.; Sakai, Y.; Takeshita, T.; Kinjo, J.; Nohara, T. *Chem. Pharm. Bull.* **1996**, *44*, 1222-1227.
30. McManus, O. B.; Harris, G. H.; Giangiacomo, K. M.; Feigenbaum, P.; Reuben, J. P.; Addy, M. E.; Burka, J. F.; Kaczorowski, G. J.; Garcia, M.L. *Biochemistry* **1993,** *32*, 6128-6133.
31. McManus, O.B.; Helms, L. M. H.; Pallanck, L.; Ganetzky, B.; Swanson, R.; Leonard, R. J. *Neuron* **1995,** *14*, 645-650.
32. Lee, M. R.; Chen, C. M.; Hwang, B. H.; Hsu, L. M. *J. Mass Spectrom.* **1999,** *34*, 804-812.
33. Applebaum, S. W.; Marco, S.; Birk, Y. *J. Agric. Food Chem.* **1969**, *17*, 618-622.
34. Su, H. C. F.; Speirs, R. D.; Mahany, P. G. *J. Econ. Ent.* **1972,** *65*, 844-847.
35. Lundbaek, J. A.; Andersen, O. S. *J. Gen. Physiol.* **1994,** *104*, 645-673.

Downloaded by STANFORD UNIV GREEN LIBR on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch015

Downloaded by CORNELL UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch016

Chapter 16

Modified Vetiver Oil: Economic Biopesticide

Kamlesh R. Chauhan[1] and Ashok K. Raina[2]

[1]Chemicals Affecting Insect Behavior Laboratory, Agricultural Research Service, U.S. Department of Agriculture, B–007, BARC-West, Beltsville, MD 20705
[2]Formosan Subterranean Termite Research Unit, SRRC, U.S. Department of Agriculture, New Orleans, LA 70124

Vetiver oil is obtained from *Vetiveria zinzanoides* L., a grass that can be found in both tropical and subtropical parts of the world. The roots of this grass on steam distillation yield an essential oil, mainly consisting of sesqiterpenes (3-4 %), sesquiterpenols (18-25%) and sesquiterpenones (7-8%). In this report we demonstrate that, sesquiterpenones and structurally related compounds exhibiting potent insecticidal activities can be enriched (35-50%) by simple chemical modification of vetiver oil. Initial studies and results utilizing modified vetiver oil for antifeedant and repellency against the Formosan subterranean termite, *Coptotermes formosanus* are discussed.

Terpenoids, especially monoterpenes and sesquiterpenes present in a number of plant-derived natural products and essential oils may have potential as insect repellents and feeding deterrents (*1,2*). Many of the insecticidal terpenes that have been tested are monoterpenoids, such as citral, eugenol, geraniol, nepetalactone, nerol, and thymol (*3-5*). However due to the limitations of bioavailability and volatility, use of monoterpenoids as biopesticides has been sparce. On the other hand there are limited reports of essential oil containing

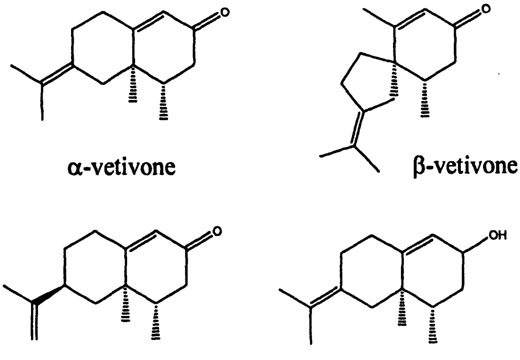
U.S. government work. Published 2006 American Chemical Society

In Natural Products for Pest Management; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

complex mixtures of sesquiterpenes possessing insecticidal or repellent activity (*6,7*). Previous literature indicating insecticidal activity of sesquiterpenones was recently reviewed (*8,9*). Ibrahim et al. (*10*) also studied two derivatives of nootkatone, namely 1,10-dihydro- and tetrahydronootkatone, and found that they were all toxic to the Formosan subterranean termite, *Coptotermes formosanus*. Of these, tetrahydronootkatone exhibited the highest toxicity. However, to develop economical biopesticides based on a single compound or natural product components is difficult. In our approach we tried to integrate available knowledge on structure-activity relationships of sesquiterpenes in commercially available essential oils, and found that Vetiver oil was the best candidate available as it is enriched with 65% sesquiterpenes.

Vetiver oil is obtained by steam distillation of the aromatic roots from the tropical grass *Vetiveria zinzanoides*, mostly from Southeast Asia where it is also called Khus. Qualities of the refined oil include a characteristic 'greenish', 'precious-woody' odor with subtle grapefruit-like nuances. The oil contains a large number of oxygenated sesqiterpenes, the most important of which are shown in Figure 1. Lately, vetiver grass has gained focus for quite another reason. Its very excessive roots are effective in preventing soil erosion in the tropics where the original forests have been largely destroyed (*11*).

Downloaded by CORNELL UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch016



α-vetivone

β-vetivone

nootkatone

vetivol

Figure 1. Sesquiterpenes in Vetiver oil

The major secondary class of sesquiterpenes present in Vetiver oil are biologically less potent (*12*) polar component vetivols or sesquiterpenols. These inactive sesquiterpene alcohols can be converted to ketones or sesquiterpenones or vetivones and khusinal by a simple oxidation processes without chromatographic separation.

Materials and Methods

Materials. Vetiver oil was purchased from Texarome, Inc. (Leakey, TX), and nootkatone (>97% crystalline) was purchased from Lancaster Synthesis (Windham, NH). Tetrahydronootkatone (95%) and modified vetiver oil mixtures were synthesized in our laboratory. Pyridinium dichromate (PDC) and palladium (Pd) used for the modification of vetiver oil and nootkatone were purchased from Aldrich Chem. Co. (Saint Louis, MO).

In the experimental approach for the modification of vetiver oil, we dissolved 10 g of vetiver oil in 100 ml of dry dichloromethane (DCM). To the resulting solution was added 1.25 weight equivalent of PDC and the mixture was stirred for 8 hr. DCM was evaporated on rotary evaporator and resulting crude mixture was redissolved in diethyl ether, filtered through a bed of celite and fluorosil (10 g). The filtered ethereal solution was concentrated on rotary evaporator and the resulting crude mixture was used for bioassay as PDC-oxidized vetiver oil. By following standard reaction procedures (*13*) 3 g of PDC oxidized vetiver oil was further modified by Pd/C catalyzed hydrogenation to generate saturated sesquiterpenones and sesquiterpenes. Proportions of sesquiterpenes, sesquiterpenones and sesquiterpenols were determined by gas chromatography (GC) and gas chromatography-mass spectroscopy (GC/MS) analyses (*12*). Simultaneously PDC-oxidation was performed on fractionated vetivols (polar fraction) of vetiver oil. Thus a total of eight compounds/mixtures were analyzed for their bioactivity in three different assays against workers of *C. formosanus* collected in standard bucket traps (*14*) set up in New Orleans, LA.

Choice Test Bioassay. We used 50 X 9 mm tight-fit Petri dishes (Falcon) with 2 filter paper disks (1 cm dia., 0.78 cm^2 area) attached at opposite ends of the dish with 1 μl DCM (Figure 2). One of the 2 disks was treated with 1, 5, or 25 $\mu g/cm^2$ of a test compound in 10 μl ethanol. Two of the test compounds were also tested at 0.2 $\mu g/cm^2$. Control disks were treated with 10 μl ethanol. Each treatment dose was replicated 4 times. Five workers were released into each dish and distribution of termites recorded every 15 minutes for 5 hours under dim red light with a time lapse video recording system (Panasonic, Japan). The data were then transcribed and statistically analyzed (*15*).

Tunneling Assay. Ten grams of moist sand was placed in each of the two 30 ml plastic cups with lids (Bio-Serv, Frenchtown, NJ). The cups were connected with a 15 cm glass tube (4mm ID). The tube was filled with sand treated with 0, 5, 25 or 125 μg of test compounds per gram of sand (Figure 3). Each treatment dose was replicated 4 times. Fifty workers and 3 soldiers were released into one of the cups and tunneling into the tubes observed after 24, 30,

Downloaded by CORNELL UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch016

36, 42, 48, 54 and 72 hours. A test was terminated once termites tunneled all the way across the tube.

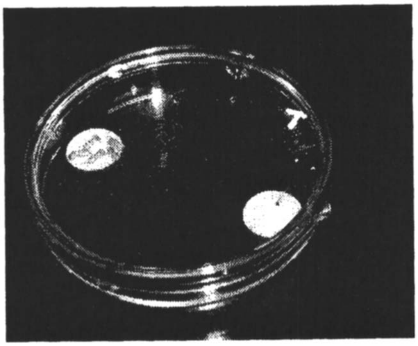


Figure 2. The setup for a choice test with C. formosanus *workers.*

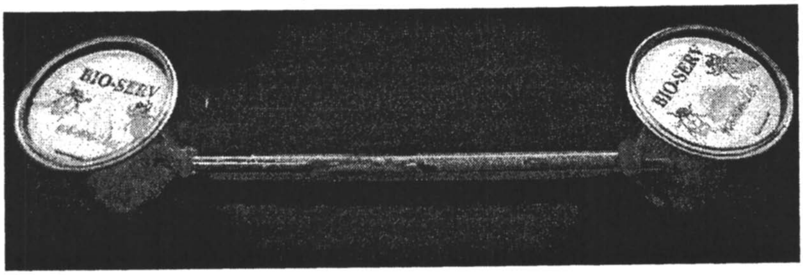


Figure 3. Experimental setup to determine tunneling through treated sand.

Feeding and Mortality. Filter paper disks (25 mm dia) were oven-dried and weighed. The test compounds were applied to the filter paper at 0, 1, 5, and 25µg/cm2. After allowing the solvent to evaporate, the filter paper was placed in a 50 X 9 mm Petri dish with a thin layer of moist sand. Termites (50 workers and 3 soldiers) were then released into the Petri dish and placed in an incubator maintained at 28^{0}C and 70% RH. After 5 days the unconsumed filter papers were removed, cleaned of debris, oven dried and weighed to determine the consumption. Live termites were counted to determine mortality. There were 4 replicates for each concentration of each chemical.

Downloaded by CORNELL UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch016

Figure 4. Feeding assay with C. formosanus *workers.*

Downloaded by CORNELL UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch016

Results and Discussion

Bioactivity of modified vetiver oil to Formosan subterranean termites is shown in Tables 1, 2, 3 and 4. Among the two valencenoids (bicyclic sesquiterpenes), vetiver oil and five modified vetiver oil mixtures tested, PDC-oxidized vetiver oil was the most repellent having consistent activity and a repellency threshold of 5 µg/cm^2 of treated filter paper.

Choice Test Results. PDC-oxidized vetiver oil was highly repellent even at 1 µg/cm^2 dose (Table 1). Nootkatone and tetrahydronootkatone were slightly repellent at a lower dose of 0.2 µg. At higher doses, these two compounds were either neutral or acted as attractants. Tetrahydronootkatone was however strongly repellent at 25 µg/cm^2.

Tunneling Results. At 0, 5, and 25 µg/g sand, the test chemicals did not affect tunneling activity and the termites tunneled through the tubes in all cases (Table 2). At 125 µg only 50% tubes with vetiver oil and PDC-oxidized vetivols were completely tunneled. For tubes treated with nootkatone and polar fraction of vetivols only 75% were completely tunneled. In case of vetiver oil, for those termites that completely crossed the tubes, it took an average of 24 hours. However, for nootkatone average time to cross the tube was 160 hours.

Feeding and Mortality Results. None of the treatments at 1 and 5 µg/cm^2 of filter paper aused a significant decrease in feeding (Table 3). At 25 µg/cm^2 PDC- oxidized vetiver oil vetivols resulted in a significant reduction in feeding. Tetrahydronootkatone caused the highest mortality at 25 µg/cm^2 dose, followed by nootkatone, vetiver oil and Pd/C reduced-oxidized vetiver oil (Table 4). None of the compounds caused any significant mortality at lower doses. Overall, tetrahydronootkatone appeared to be the best treatment.

Downloaded by CORNELL UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch016

Table 1. Distribution of *C. formosanus* workers on treated vs. control filter paper disks treated with various test chemicals at three concentrations in a choice test.*

			% Termites on control and treated disks		
	Treatment		1 µg/cm^2	5 µg/cm^2	25µg/cm^2
1	Nootkatone	control	17.8 ± 20.6	19.9 ± 6.3	8.1 ± 3.4
		treatment	28.5 ± 25.3	7.6 ± 5.4	18.3 ± 2.7
2	Tetrahydronootkatone	control	4.0 ± 0.8	20.4 ± 8.2	16.4 ± 2.2
		treatment	15.0 ± 12.0	10.7 ± 1.7	10.5 ± 0.3
3	Vetiver oil	control	24.0 ± 7.5	23.0 ± 8.6	4.5 ± 4.2
		treatment	4.5 ± 3.3	8.8 ± 2.5	10.4 ± 0.5
4	Non-polar fr.	control	15.9 ± 7.0	13.1 ± 9.9	23.3 ± 5.1
		treatment	25.9 ± 10.2	22.9 ± 10.5	14.8 ± 7.0
5	Polar fr. (vetivols)	control	11.2 ± 3.1	30.2 ± 4.0	22.9 ± 13.7
		treatment	22.9 ± 8.5	7.4 ± 3.5	9.3 ± 3.5
6	PDC oxidized vetiver oil	control	31.0 ± 8.9	31.7 ± 9.0	24.5 ± 4.8
		treatment	2.1 ± 0.9	5.9 ± 2.4	2.1 ± 1.0
7	Pd/C reduced oxi. Vetiver oil	control	21.9 ± 5.4	13.6 ± 6.3	20.7 ± 3.7
		treatment	16.9 ± 8.4	28.1 ± 6.8	25.7 ± 9.6
8	PDC oxidized vetivols	control	13.1 ± 9.6	7.9 ± 4.7	6.2 ± 2.8
		treatment	11.2 ± 9.0	8.1 ± 3.1	7.1 ± 6.2

* Values are averages ± SE, N = 4.

Downloaded by CORNELL UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch016

Table 2. Tunneling and time to tunnel (hours) by *C. formosanus* workers through sand treated with various test chemicals at three concentrations.*

	Treatment	% Crossed at dose (µg/g)			
		0	5	25	125
1	Nootkatone	100	100	100	75
2	Tetrahydronootkatone	100	100	100	100
3	Vetiver oil	100	100	100	50
4	Non-polar fr.	100	100	100	100
5	Polar fr. (vetivols)	100	100	100	75
6	PDC oxidized vetiver oil	100	100	100	100
7	Pd/C reduced oxi. Vetiver oil	100	100	100	100
8	PDC oxidized vetivols	100	100	100	50

	Treatment	Average hours taken to cross at dose (µg/g)			
		0	5	25	125
1	Nootkatone	24 ± 0.0	24 ± 0.0	24 ± 0.0	160 ± 27.7
2	Tetrahydronootkatone	30 ± 6.0	30 ± 6.0	24 ± 0.0	78 ± 6.0
3	Vetiver oil	24 ± 0.0	24 ± 0.0	24 ± 0.0	24 ± 0.0
4	Non-polar fr.	30 ± 6.0	24 ± 0.0	24 ± 0.0	24 ± 0.0
5	Polar fr. (vetivols)	42 ± 11.5	36 ± 6.9	54 ± 22.7	40 ± 6.9
6	PDC oxidized vetiver oil	24 ± 0.0	24 ± 0.0	36 ± 6.9	24 ± 0.0
7	Pd/C reduced oxi. Vetiver oil	24 ± 0.0	24 ± 0.0	30 ± 6.0	24 ± 0.0
8	PDC oxidized vetivols	24 ± 0.0	72 ± 21.9	48 ± 13.8	24 ± 0.0

*Values are averages ± SE, N = 4.

Table 3. Consumption (mg) by *C. formosanus* workers of filter paper treated with various test chemicals at three different concentrations.*

	Treatment	Average feeding (mg)			
		Control	1 µg/cm^2	5 µg/cm^2	25 µg/cm^2
1	Nootkatone	22.7 ± 2.4	22.7 ± 2.7	13.3 ± 2.3	19.3 ± 1.8
2	Tetrahydronootkatone	15.7 ± 3.1	24.7 ± 3.6	13.9 ± 3.1	10.1 ± 3.0
3	Vetiver oil	18.8 ± 2.3	18.9 ± 3.7	20.5 ± 3.3	20.0 ± 3.1
4	Non-polar fr.	18.2 ± 2.9	16.0 ± 1.0	21.1 ± 2.0	12.9 + 2.4
5	Polar fr. (vetivols)	19.5 ± 4.2	16.1 ± 2.3	18.2 ± 5.4	15.7 ± 3.0
6	PDC oxidized vetiver oil	22.3 ± 2.0	17.1 ± 3.8	16.1 ± 3.5	10.1 ± 1.8
7	Pd/C reduced oxi. Vetiver oil	19.1 ± 1.9	14.5 ± 2.8	19.3 ± 3.1	17.8 ± 2.0
8	PDC oxidized vetivols	11.8 ± 4.1	17.2 ± 3.1	15.7 ± 4.6	9.1 ± 2.1

*Values are averages ± SE, N = 4.

Table 4. Mortality of *C. formosanus* workers caused by exposure to/feeding on filter paper with test chemicals at three different concentrations.*

	Treatment	Average mortality			
		Control	1 µg/cm^2	5 µg/cm^2	25 µg/cm^2
1	Nootkatone	8.2 ± .0	4.6 ± 1.6	6.4 ± 3.4	20.0 ± 4.4
2	Tetrahydronootkatone	2.7 ± 2.0	6.4 ± 2.1	5.5 ± 2.9	30.0 ± 4.9
3	Vetiver oil	5.4 ± 1.9	0.9 ± 1.0	6.4 ± 3.0	10.0 ± 3.3
4	Non-polar fr.	4.5 ± 2.5	3.7 ± 1.9	1.8 ± 1.2	4.5 ± 3.2
5	Polar fr. (vetivols)	4.6 ± 2.7	2.8 ± 1.2	5.7 ± 1.0	1.8 ± 1.2
6	PDC oxidized vetiver oil	2.8 ± 1.2	4.6 ± 1.6	5.5 ± 1.9	5.5 ± 1.9
7	Pd/C reduced oxi. Vetiver oil	4.6 ± 1.6	2.8 ± 1.2	5.5 ± 1.0	10.0 + 1.9
8	PDC oxidized vetivols	2.7 ± 3.0	3.6 ± 4.0	2.8 ± 1.2	3.7 ± 2.0

*Values are averages ± SE, N = 4.

Downloaded by CORNELL UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch016

Summary

Modified vetiver oil, enriched with sesquiterpenones (PDC-oxidized) exhibited potent repellency to Formosan subterranean termites at different concentrations in choice test while tetrahydronootkatone exhibited significant repellency at only the higher dose. Tetrahydronootkatone caused the highest mortality at 25 µg/cm^2 dose, followed by nootkatone, vetiver oil and Pd/C reduced-oxidized vetiver oil. Overall tetrahydro nootkatone appeared to be the best treatment in feeding and mortality experiments. At 125 µg dose only 50% tubes with vetiver oil and PDC oxidized vetivols were completely tunneled. At 125 µg dose 75% tubes treated with nootkatone and vetivols were completely tunneled. At the same dose, for the nootkatone the average time to cross the tube was 160 hrs. Sesquiterpenoids known in nature outnumber monoterpenoids (*16*) and have low mammalian toxicity. The most important advantage for sesquiterpenoid as repellents is their persistence and low volatility (*17*).

By simple modifications we can enhance desired bioactivities of natural product extracts or essential oils without isolating individual components. In the case of vetiver oil, the insect repellent or deterrent activity is due to the presence of sequiterpenones (cyclic ketones); we can enrich these sequiterpenones by oxidizing relatively inactive sesquiterpenols (cyclic alcohols). Thus modified vetiver oil, enriched with sequiterpenones would be a product of an economical and environmentally sound bio-pesticide.

References

1. Isman, M. *Pestic. Outlook.* **1999,** May, 68–72.
2. Franzios, G.; Mirotsou, M.; Hatziapostolou, E.; Kral, J.; Scouras, Z. G.; Mavraganitsipidou, P. *J. Agric. Food Chem.* **1997,** *45,* 2690–2694.
3. Rice, P. J.; Coats, J. R. *J. Econ. Entomol.* **1994,** *87.* 1172–1179.
4. Cornelius, M. L.; Grace, J. K.; Yates J. R. *J. Econ. Entomol.* **1997,** *90,* 320–325.
5. Peterson, C. J.; Ems-Wilson, J. *J. Econ. Entomol.* **2003,** *96,* 1275-1282.
6. Jain, S. C.; Nowicki, S.; Eisner, T.; Meinwald, J. *Tetrahedron Lett.* **1982,** 23, 4629-4642.
7. Sharma, R. N.; Tungikar, V. B.; Pawar, P. V.; Vartak, P. H. *Insect Sci. Appl.* **1994,** *15,* 495–498.
8. Maistrello, L.; Henderson, G.; Laine, R. A. *J. Econ. Entomol.* **2001,** *94,* 1523–1537.
9. Zhu, B. C. R.; Henderson, G.; Sauer, A. M.; Yu, Y.; Crowe, W.; Laine, R. A. *Journal of Chemical Ecology,* **2000,** *29,* 2695-2701
10. Ibrahim, S. A.; Henderson, G.; Zhu, B. C. R.; Fei, H.; Laine, R. A. *J. Econ. Entomol.* **2004,** *97,* 102-111.
11. *Vetiver Grass, A Thin Green Line Against Erosion*; Vietmeyer, N. D.; Ruskin, F. R.; Eds.; National Academy Press: Washington D.C., **1993.**
12. Chauhan, K. R.; Raina, A. Abstracts of Papers, 227th ACS National Meeting, Anaheim, CA, United States, March 28-April 1, 2004 (2004), AGRO-004.
13. Chauhan, K. R.; Bhatt, R. K.; Falck, J. R.; Capdevila, J. H. *Tetrahedron Lett.,* **1994,** *35,* 1825-1828.
14. Su, N.-Y.; Scheffrahm, R. H. *Sociobiol.* **1986,** *12,* 299-304.
15. SAS Institute. **1996**. SAS for Windows, version 6.12 SAS User's Guide, Cary, NC.
16. Harborne, J. B. In *Ecological Chemistry and Biochemistry of Plant Terpenoids*; Harborne, J. B.; Tomas-Barberan, F. A., Eds.; Clarindon Press: Oxford, UK, 1991; pp 399-426.
17. Zhu, B. C. R.; Henderson, G.; Chen, F.; Fei, H.; Laine, R. A. *J. Chem. Ecol.* **2001,** *27,* 1617-1625.

Downloaded by CORNELL UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch016

Downloaded by UNIV OF GUELPH LIBRARY on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch017

Chapter 17

Engineering Natural Products for Crop Resistance to Parasitic Weeds

James H. Westwood

**Department of Plant Pathology, Physiology, and Weed Science,
410 Price Hall, Virginia Polytechnic Institute and State University,
Blacksburg, VA 24061**

Parasitic weeds of the genus *Orobanche* (broomrapes) pose a challenging problem for agriculture because they are highly destructive and difficult to control. Many strategies have been used to limit crop damage from parasites, but all are limited by incomplete efficacy or prohibitive economics. An ideal solution would be the development of *Orobanche*-resistant crops, but to date little progress has been made toward this goal. As understanding of host-parasite interactions becomes more sophisticated, it is possible to consider genetically engineering parasite resistance into host crops. One approach would be to engineer the host to produce a toxin that selectively inhibits growth of the parasite, but few candidate toxins are known. Natural products represent an important source of potential anti-parasite compounds, and this chapter will describe one such example, sarcotoxin IA, an antibacterial peptide originally isolated from the flesh fly (*Sarcophaga peregrina*). Transgenic tobacco plants expressing this peptide have shown enhanced resistance to *Orobanche* parasitism and this may represent a new model for engineering resistance to parasitic weeds.

In Natural Products for Pest Management; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

Introduction

Parasitic plants are among the world's most destructive weeds (*1*). Unlike non-parasitic weeds, which simply compete with crops for resources such as light, water, and nutrients, parasitic weeds tap directly into the crop, removing resources and altering host physiology. Parasitic plants connect to their hosts through a specialized structure, the haustorium, which forms a physiological bridge between the two plants. The haustorium serves as a conduit for the removal of water, minerals, and photosynthates from the host, thereby draining it of resources it needs to grow and reproduce (*2*). In addition to nutrient acquisition, parasite consumption of host water may cause the host to experience drought even when environmental water levels are not limiting. The resulting water conservation efforts by the host only serve to suppress its own photosynthesis and arrest growth. Parasites also may disrupt hormone balance in the host, causing deformities and reallocation of resources away from shoots and fruits.

Over 1% of plant species are parasitic, and parasitism in plants has evolved multiple times, with 18 botanical families having parasitic members (*3*). The multiple evolutionary origins of parasitism account in part for the large diversity among parasite species in growth form, host preference, and reproductive strategy, but they all have in common the haustorium, a structure unique to parasitic plants (*4*). This chapter will focus on the genus *Orobanche*, which is part of the economically important and well-charactacterized Orobanchaceae that also includes *Striga* (witchweed) species. The ideas presented may be applicable to other parasitic lineages, although caution is called for when generalizing across widely divergent groups of parasites.

Downloaded by UNIV OF GUELPH LIBRARY on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch017

Orobanche

Impact of Orobanche

The *Orobanche* (broomrape) species are obligate holoparasites that attack the roots of many economically-important crops throughout the semiarid regions of the world, especially the Mediterranean and Middle East, where they are endemic. However, these plants have been spread thoughout the world and have been reported in Africa, Australia, New Zealand, Japan and the U.S. (*1, 5-7*). *Orobanche* species attack members of the Solanaceae, Fabaceae, Compositae and more than 30 other food and ornamental crops, causing severe losses in yield and quality (*1*). The genus *Orobanche* has more than 100 species, with five (*O. aegyptiaca*, *O. ramosa*, *O. minor*, *O. cernua*, and *O. crenata*), being considered significant parasites of crops that include members of the Solanaceae, Fabaceae, Compositae, Cruciferae and Umbelliferae families (*1*).

Orobanche species lack developed leaves (Figure 1A) and chlorophyll, so have evolved adaptations to maximize their likelihood of forming an attachment to a suitable host. For example, these parasites rely on detection of host chemical signals to stimulate seed germination and haustorium formation (*8*). These early stages of parasite development are critical to survival, because a germinated seedling that either fails to contact or connect to a host will exhaust its energy reserves and die. Following successful attachment to a host, the *Orobanche* tissue adjacent to the host root swells to form a bulbous structure called a tubercle (Figure 1B-C). This gives rise to short, secondary roots that are capable of forming new host attachments (Figure 1D). After two to four weeks of growth, a floral meristem is produced, which emerges above ground to flower and disseminate seeds. A single plant may produce hundreds of thousands of seeds, which may remain viable in the soil for over 10 years (*1*).

Downloaded by UNIV OF GUELPH LIBRARY on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch017

Current control strategies

Parasitic weeds such as *Orobanche* and *Striga* are difficult to control because they are closely associated with the host root and are concealed underground for most of their life cycle. Cultural practices (planting date, soil fertility, and crop rotations that include non-host crops) can help manage *Orobanche* populations, but have inconsistent effectiveness (*9*). Currently in Israel and throughout the Middle East, the most effective control method used is soil fumigation with methyl bromide (*10*). However, this is expensive, laborious, and hazardous to the environment, and methyl bromide use is being phased out by international agreement to protect the global environment. Herbicides have been evaluated for many years (*11*), and certain translocated herbicides can be effective when used in crops that can tolerate low rates of the chemicals (*9*). The development of herbicide-resistant crops allows higher doses of herbicide to be safely translocated through the host plant to exert its toxic effect specifically on the parasite (*12, 13*) and may lead to wider use of herbicides in *Orobanche* control. However, this approach depends on commercial availability of herbicide-resistant varieties for affected crops, and this has not yet been realized. The technology closest to commercial resease is imidazolinone-resistant maize for control of *Striga* in Africa (*14*).

The best long-term strategy for limiting damage by *Orobanche* is the development of parasite-resistant crops (*15, 16*). Such crops would have the advantage of not requiring chemical inputs, thereby avoiding costs of chemicals and the risks of crop injury associated with their application. Resisitant crops could also reduce the parasite seed populations if seeds were stimulated to germinate normally yet fail to successfully parasitize their host. Unfortunately, for most parasitized crops resistant germplasm either does not exist or resistance traits have been difficult to transfer to cultivated viarieties. In many cases where resistance has been obtained, it has not been durable over time or different geographic locations (*17*).

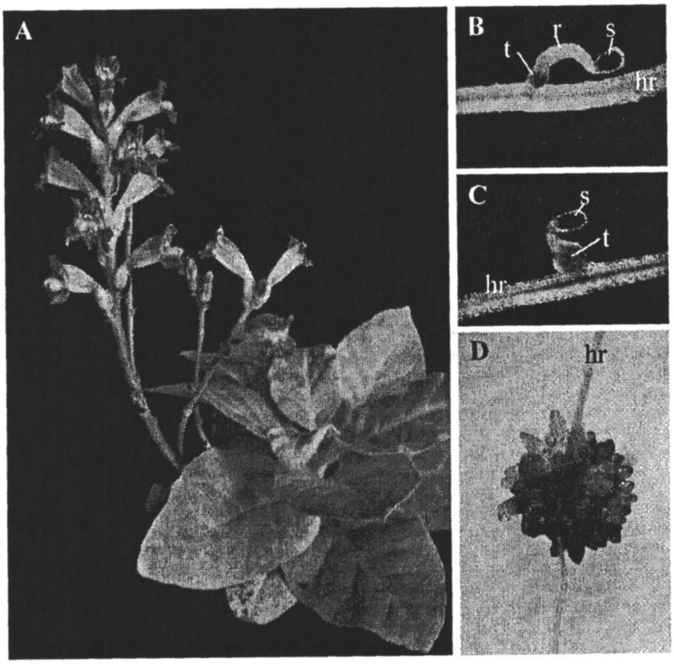


Figure 1. Orobanche aegyptiaca growing on tobacco. A, Floral shoot of the parasite emerging from the soil at the base of the host. B, An early stage in parasite development showing established tubercle just beginning to form outside the host root. C, Expanding tubercle. D, Mature tubercle prior to development of the floral shoot, but showing secondary roots. Parasite seed coat (s), radicle (r), tubercle (t) and the host root (hr) are indicated.

Downloaded by UNIV OF GUELPH LIBRARY on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch017

Physiology of the host-parasite connection

The anatomy and physiology of the host-parasite connection is important because it determines how the parasite obtains resources from the host, and conversely, how host resistance may be manipulated to block the transfer of resources. Anatomically, xylem connections between host and parasite appear to lack any barriers to water movement. Scanning electron micrographs of sorghum parasitized by *S. hermonthica* show open-ended parasite xylem vessels protruding through pits in the walls of the host vessels, forming a continuous connection between xylem of the two plants (*18*). This is likely to be similar for *Orobanche*, but in this case xylem links are supplemented by connections between host and parasite sieve elements (*19*), suggesting a symplastic continuum as well.

Physiological experiments support these findings, and the host-parasite translocation of radiolabeled sugar (*20*), photosynthates and inorganic phosphate (*21*), and herbicides (*22, 23*) have been documented. *Orobanche* obtains these resources by creating a strong osmotic gradient (*24, 25*), such that small molecules move readily into the parasite. Until recently, little was known about *Orobanche* uptake of larger molecules such as proteins. Another parasitic plant, dodder (*Cuscuta reflexa*), was shown to accumulate green fluorescent protein (GFP) produced in phloem companion cells of a tobacco host (*26*). However, dodder is a shoot parasite with different anatomy and haustorial connections as evidenced by its ability to transmit viruses among host plants, a feature that has not been reported for root parasites such as *Orobanche*.

Recently, the translocation of GFP and other macromolecules has been demonstrated from tobacco to *O. aegyptiaca* (*27*). This work studied apoplastic movement of macromolecules from host to parasite and documented the transfer of proteins as large as 27 kDa and fluorescent dextrans up to 70 kDa. This data supports a model in which *Orobanche* indiscriminately draws water and solutes (including macromolecules) from the host xylem.

Natural products for control of *Orobanche*

Natural products represent a potential source of compounds that may be used to control *Orobanche*. For example, 7-hydroxylated simple coumarins such as scopoletin have been implicated in sunflower resistance to *O. cernua* (*28*). It is likely that other natural products may also act as allelochemicals or phytoalexins, and thereby prove valuable in enhancing reistance. Such products need not be restricted to those produced in plant roots during parasitism, but could be transferred by genetic engineering from other organisms. Nevertheless, the greatest challenge remains identifying products with potential activity against the parasite.

As suggested by scopoletin in sunflower, parasite-resistant crop varieties may provide leads on natural plant products that could be manipulated to enhance defense. Unfortunately, in most cases of resistance to *Orobanche*, the precise mechanism remains poorly characterized. Host defense mechanisms that have been demonstrated or suggested include low germination stimulant production, structural reinforcement of cell walls, production of phytoalexins, and host root necrosis due to the hypersensitive response (Reviewed in *29*).

Downloaded by UNIV OF GUELPH LIBRARY on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch017

Natural products involved in host defense responses will be an important area of investigation, but *Orobanche* appears to succeed in parasitizing some hosts despite evidence that the host's defenses are activated (*30*).

The Earth's organisms collectively contain a vast array of bioactive compounds. Many of these have been found to be useful, and have been developed as solutions to problems in quite a different context from which they were derrived. One example of this with relevance to parasitic weeds is sarcotoxin IA, an antibiotic peptide from insects that appears to have value as a toxin against *Orobanche.*

Sarcotoxin

Potential use of sarcotoxin IA for crop improvement

The potential value of sarcotoxin IA as an *Orobanche* toxin was first demonstrated using recombinant sarcotoxin IA synthesized by *Saccharomyces cerevisiae* (*31*). When applied to *O. aegyptiaca* seeds at concentrations of 10 µM or higher, sarcotoxin IA inhibited germination and radicle elongation (Aly and Plakhine, unpublished results).

Sarcotoxin IA is a 39-residue peptide of the cecropin family, a class of small, anti-microbial polypeptides that have been isolated from insects (*32*), including the flesh fly, *Sarcophaga peregrina* (*33*). The mode of action of sarcotoxin IA and related cecropins is by disruption of bacterial cell membranes, causing a loss of electrochemical potential (*34-37*). Selectivity of these toxins for bacteria is attributed to the positively-charged hydrophilic end of the peptide that associates preferentially with bacterial membranes, which carry a more negative charge than eukaryotic cells (*38*). Therefore, the toxins disrupt bacterial cells at concentrations one to two orders of magnitude lower than those that disrupt mammalian or plant cells (*39, 40*).

The differential sensitivity of prokaryotic and eukaryotic cells to sarcotoxin IA suggested that it may be used to generate transgenic plants with enhanced pathogen resistance. This has been tested, and transgenic tobacco expressing sarcotoxin IA showed enhanced resistance to bacterial (*Erwinia carotovora* subsp. *carotovora* and *Pseudomonas syringae* pv. *tabaci*) and fungal (*Rhizoctonia solani* and *Pythium aphanidermatum*) pathogens (*41, 42*). In addition, other cecropin peptides have been used to confer enhanced resistance in rice and potato. Transgenic rice expressing cecropin B showed increased resistance to *Xanthomonas oryzae* pv. *oryzae* infection (*43*), and potato carrying a cecropin-melittin cationic peptide chimera had enhanced resistance to fungi (*Phytophthora cactorum* and *Fusarium solani*) and a bacterium (*Erwinia carotovora*).

Downloaded by UNIV OF GUELPH LIBRARY on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch017

The use of sarcotoxin IA against a parasitic plant is surprising considering that the level of selectivity between cells of the host and those of the parasite should be less than that between plants and bacteria. Nevertheless, transgenic tobacco plants expressing sarcotoxin IA under the control of the constitutive, root-specific TobRB7 promoter (*44, 45*) showed reduced parasitism by *Orobanche* (Aly et al., unpublished data). However, resistance in these plants was incomplete, perhaps due to the low level of expression driven by the TobRB7 promoter.

In order to enhance sarcotoxin IA levels, studies were conducted using an inducible promoter to concentrate transgene expression specifically at the time and location of parasite penetration (*27*). Transgenic plants were created by fusing the sarcotoxin IA gene to the promoter from *HMG2*, a defense-specific isogene of 3-hydroxy-3-methylglutaryl CoA reductase from tomato, which has been shown to have strong, sustained expression in host roots at the site of *Orobanche* entry (*46*). This promoter exhibits a number of desirable features with respect to engineering parasite resistance in that it is induced rapidly following penetration of the host root, and expression occurs specifically in the area immediately surrounding the point of attachment and continues for at least the first four weeks of parasite development. The *HMG2* promoter is not normally active in healthy tissues, with the exception of a few specific sites such as cotyledons, trichomes of young leaves, sites of lateral root initiation, and developing anthers (*47*).

Downloaded by UNIV OF GUELPH LIBRARY on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch017

Tobacco expressing sarcotoxin IA show enhanced Orobanche *resistance*

Transgenic tobacco plants containing the *HMG2:sarcotoxin IA* fusion appeared normal and were fertile (*48*). Two lines, L03 and L07, were selected for characterization and the presence of the transgene was confirmed by DNA hybridization analysis. This indicated that L03 contained one copy of the transgene and L07 contained at least three copies, but both expressed sarcotoxin IA mRNA and contained approximately equal levels of the protein.

When challenged in soil inoculated with *O. aegyptiaca* seeds, tobacco lines L03 and L07 had significantly higher biomass accumulation than non-transgenic tobacco lines (Figure 2A). Although this difference was remarkable, the resistance was incomplete as shown by the inoculated transgenic plants accumulating less biomass than equivalent non-inoculated plants. When grown in the absence of *O. aegyptiaca*, tobacco lines containing the sarcotoxin IA gene showed no differences in growth compared to non-transformed lines, indicating that the transgene itself did not cause an obvious productivity penalty to the tobacco (Figure 2 A).

Downloaded by UNIV OF GUELPH LIBRARY on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch017

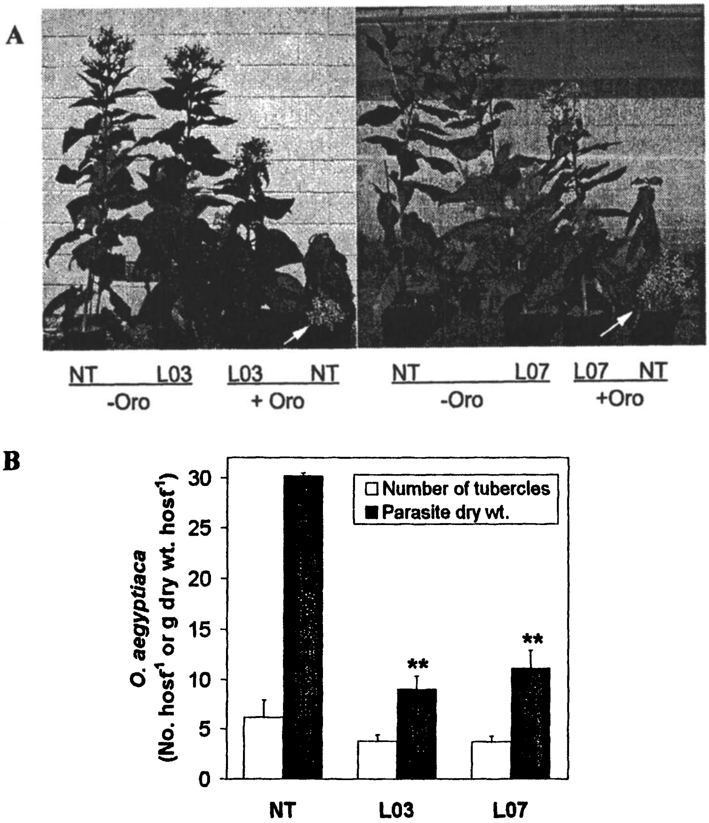


*Figure 2. Response of HMG2:SARCO transformed tobacco plants to O. aegyptiaca in a soil-based assay. A, Photo showing phenotypes of NT, L03, andL07 plants in non-inoculated soil (-Oro) and soil inoculated with O. aegyptiaca (+Oro). Arrows show O. aegyptiaca flowers emerged on NT tobacco. B, Numbers and dry weights of O. aegyptiaca parasitizing the NT, L03, and L07 lines. Data aret means ± SE. **indicates means different from NT by Student's t-test, α = 0.01. Figure is modified and reprinted (48) with kind permission of Springer Science and Business Media. Copyright 2005.*

The effect of sarcotoxin IA expression on the parasite generally reflected a post-attachment resistance mechanism. The numbers of *O. aegyptiaca* tubercles that established on transgenic hosts did not differ significantly from those on non-transformed plants, but the biomass of parasites was significantly lower on lines L03 and L07 compared to non-transformed controls (Figure 2B). Furthermore, parasites growing on the transformed hosts frequently exhibitited abnormal or stunted development (*48*). These results support a hypothesis by which sarcotoxin IA affects parasite growth after attachment has occurred and the parasite has started to consume host resources, rather than affecting the process of initial parasite attachment. This is expected because the inducible *HMG2* promoter controlling sarcotoxin IA expression should not be active until after the parasite has penetrated the host root (*46*). To assess the robustness of this mechanism against other *Orobanche* species, transgenic lines were also challenged with *O. ramosa* and similar effects on parasite growth were observed (*48*).

Some parasites growing on transgenic plants were able to develop to the point of producing floral shoots, and these may represent escapes from the effect of sarcotoxin IA. The interaction between host and parasite is complex and is influenced by the host size, the number and size of the parasites, and other factors that affect relative sink strengths in host and parasite (*2*), so even the presence of a few parasites appears to impact growth of the host.

Downloaded by UNIV OF GUELPH LIBRARY on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch017

Possible mechanisms of action of sarcotoxin IA against Orobanche

In order to optimize the effect of sarcotoxin IA on *Orobanche* parasitism, it will be important to understand its mechanism of action. Although it is difficult to draw definitive conclusions based on current information, it is interesting to consider this subject in light of what is known about sarcotoxin IA and the host-parasite interaction.

The paradox of sarcotoxin IA as an anti-*Orobanche* plant agent is that it is known to preferentially disrupt bacterial membranes as compared to eukaryotic membranes (*36, 37*). Why would it inhibit growth of *Orobanche* more than the host whan both are eukaryotes? Two hypothesized mechanisms could explain this: 1) sarcotoxin IA acts on cells of the host root to induce host defenses or otherwise disrupt host metabolic activity important in the parasite connection, or 2) sarcotoxin IA may be translocated into *Orobanche* and be toxic directly to parsite cells. Because these hypotheses are not mutually exclusive, it is also possible that both may contribute to the observed resistance. Sarcotoxin IA will injure plant cells, so the determining factor for toxicity is most likely to be the localization of accumulated peptide.

The findings that macromolecules up to 27 kDa in size readily move from host to parasite through xylem connections (*27*) argues that movement of the 4 kDa sarcotoxin IA should be possible. The sarcotoxin IA constructs used in the above experiments contained a signal peptide to target the protein for secretion to the extracellular space, where it should be free to move into the parasite with the bulk flow of water. *Orobanche* plants are well characterized as strong sinks on their hosts (*20, 23*), so the potential exists for *Orobanche* to accumulate macromolecules such as sarcotoxin IA.

Regardless of the site of sarcotoxin IA localization, its inhibitory effect is likely to be concentration-dependent. Ohashi and colleagues (*41, 42, 49*) identified promoter strength and sarcotoxin IA stability as key features in obtaining sufficient accumulation of the peptide to confer bacterial and fungal resistance on transgenic tobacco. The *HMG2* promoter used in the *Orobanche* study appears to be strongly induced by *Orobanche* parasitism, although its expression is restricted to a small region of host root around the point of parasite entry (*46*).

Downloaded by UNIV OF GUELPH LIBRARY on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch017

Advantages and risks of sarcotoxin IA

Because sarcotoxin IA and other cecropins target membranes rather than specific receptors, they are effective against a wide range of bacteria, and have been recognized as potentially useful tools for enhancing plant resistance to microbial pathogens (*42*). Although it is not certain that they act on plants such as *Orobanche* through the same mechanism, membranes of the parasite are an attractive target for a control strategy. The relianace of *Orobanche* on establishing a strong osmotic gradient relative to the host is a key feature of its ability to obtain resources, so any disruption of membrane integrity would be expected to be highly deleterious to the parasite.

Sarcotoxin IA has favorable toxicological properties in that it is a protein that does not persist long in the environment or human digestive system (*50*). Also, homologs of sarctoxin IA are found in insects and mammals, so this class of compounds may be one to which humans are already exposed. In addition, when its expression is controlled by an inducible promoter such as *HMG2*, the levels of sarcotoxin IA that accumulate in consumed plant parts should be relatively low.

Potential risks of sarcotoxin IA use for *Orobanche* control arise from the bactericidal and fungicidal properties expressed in transgenic plants. If sarcotoxin IA has such broad activity, plants expressing sarcotoxin IA may have superior fitness when faced with a variety of pathogens and the risk would be that the gene conferring such a fitness benefit may escape in outcrosses with weedy relatives of the crop. As with any transgenic crop, the potential for gene flow will need to be evaluated and fitness advantages attenuated if necessary. The development of resisitance in the pathogen population will also threaten to make the trait obsolete. Proper management of transgenic crops and pests such that multiple control methods are employed will be essential for maintaining the efficacy of the resistance mechanism.

This area of research is still young, and it remains an open question whether plants expressing sarcotoxin IA will prove to be sufficiently useful in deterring parasitism that they reach the point of commercial release. Several important refinements are needed to achieve sufficient levels of *Orobanche* resistance needed in the field. Nevertheless, this compound represents an important lead in developing engineered resistance to parasitic weeds and demonstrates the value in exploring natural products for novel uses.

Acknowledgements

This chapter is based on collaborative work with N. Hamamouch, R. Aly, C. Cramer, and I. Banner. This research was supported by Research Grant No. IS-3048-98 from BARD, The United States – Israel Binational Agricultural Research and Development Fund. Additional support was provided by USDA NRI award 2001-35320-10900 and USDA Hatch Project No. 135657.

Downloaded by UNIV OF GUELPH LIBRARY on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch017

Literature Cited

1. Parker, C.; Riches, C. R., *Parasitic Weeds of the World: Biology and Control*; CAB International: Wallingford, 1993; pp 111-164.
2. Graves, J. D. In *Parasitic Plants*; Press, M. C.; Graves, J. D., Eds.; Chapman & Hall: London, 1995; pp 206-225.
3. Nickrent, D. L.; Duff, R. J.; Colwell, A. E.; Wolfe, A. D.; Young, N. D.; Steiner, K. E.; de Pamphilis, C. W. In *Molecular Systematics of Plants II: DNA sequencing*; Soltis, D. E.; Soltis, P. S.; Doyle, J. J., Eds.; Kluwer Academic Publishers: Boston, 1998; pp 211-241.
4. Kuijt, J., *The Biology of Parasitic Flowering Plants*; University of California Press: Berkeley, 1969; pp 158-190.
5. James, R. W. *New Zealand Tobacco Growers Journal* **1976,** *May*, 7-8.
6. Hassan, E. A. In *Comptes rendu 6ème Symposium Méditerranéen EWRS*, Maillet, J., Eds. ENSA: Montpellier, France, 1998; pp 13-15.
7. Musselman, L. J. In *Biology and management of Orobanche, Proceedings of the Third International Workshop on Orobanche and related Striga research*, Pieterse, A. H.; Verkleij, J. A. C.; ter Borg , S. J., Eds. Royal Tropical Institute: Amsterdam, The Netherlands, 1994; pp 27-35.
8. Press, M. C.; Graves, J. D.; Stewart, G. R. *Plant Cell Environ.* **1990,** *13*, 91-104.
9. Goldwasser, Y.; Kleifeld, Y. In *Weed Biology and Management*; Inderjit, Eds.; Kluwer Academic Publishers: Dordrecht, 2004; pp 439-566.

10. Jacobsohn, R. In *Biology and Management of Orobanche, Proc. 3rd Int. Workshop on Orobanche and Related Striga Res.*, Pieterse, A. H.; Verkleij, J. A. C.; ter Borg, S. J., Eds. Royal Tropical Inst.: Amsterdam, The Netherlands, 1994; pp 652-658.
11. Foy, C. L.; Jain, R.; Jacobsohn, R. *Rev. Weed Sci.* **1989,** *4*, 123-152.
12. Joel, D. M.; Kleifeld, Y.; Losner-Goshen, D.; Gressel, J. *Nature* **1995,** *374*, 220-221.
13. Surov, T.; Aviv, D.; Aly, R.; Joel, D. M.; Goldman-Guez, T.; Gressel, J. *Theor. Appl. Genet.* **1998,** *96*, 132-137.
14. Kanampiu, F. K.; Ransom, J. K.; Gressel, J. *Crop Prot.* **2001,** *20*, 885-895.
15. Cubero, J. I. In *Progress in Orobanche research*, Wegmann, K.; Musselman, L. J., Eds. Eberhard-Karls-Univrersitat, Tubingen, FRG: 1991; pp 257-277.
16. Ejeta, G.; Butler, L. G.; Hess, D. E.; Vogler, R. K. In *Proceedings of the 5th International Symposium of Parasitic Weeds*, Ransom, J. K.; Musselman, L. J.; Worsham, A. D.; C., P., Eds. CIMMYT: Nairobi, Kenya, 1991; pp 539-544.
17. Alonso, L. C. In *Proceedings of the Fourth International Workshop on Orobanche*, Wegmann, K.; Musselman, L. J.; Joel, D. M., Eds. Institute for Wheat and Sunflower "Dobroudja": Albena, Bulgaria, 1998; pp 233-257.
18. Dörr, I. *Ann. Bot.* **1997,** *79*, 463-472.
19. Dörr, I. In *Advances in Parasitic Plant Research*, Moreno, M. T.; Cubero, J. I.; Berner, D.; Joel, D.; Musselman, L. J., Eds. Junta de Andalucia: Cordoba, Spain, 1996; pp 195-201.
20. Aber, M.; Fer, A.; Sallé, G. *Z. Pflanzenphysiol. Bd.* **1983,** *112*, 297-308.
21. Ismail, A. M. A.; Obeid, M. *Weed Res.* **1976,** *16*, 87-92.
22. Muller, F.; Distler, B. In *Progress in Orobanche Research*, Wegmann, K.; Musselman, L. J., Eds. Eberhard-Karls-Universität: 1991; pp 226-233.
23. Nandula, V. K.; Foy, C. L.; Orcutt, D. M. *Weed Sci.* **1999,** *47*, 486-491.
24. Harloff, H. J.; Wegmann, D. *J. Plant Physiol* **1993,** *141*, 513-520.
25. Stewart, G. R.; Press, M. C. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1990,** *41*, 127-151.
26. Haupt, S.; Oparka, K. J.; Sauer, N.; Neumann, S. *J. Exp. Bot.* **2001,** *52*, 173-177.
27. Hamamouch, N. Ph.D. thesis, Virginia Tech, Blacksburg, VA, 2004.
28. Serghini, K.; Pérez de Luque, A.; Castejón-Muñoz, M.; García-Torres, L.; Jorrín, J. V. *J. Exp. Bot.* **2001,** *52*, 2227-2234.
29. Westwood, J. H. In *Weed Biology and Management*; Inderjit, Eds.; Klewer Academic Publishers: Dordrecht, 2004; pp 177-198.
30. Griffitts, A. A.; Cramer, C. L.; Westwood, J. H. *Weed Sci.* **2004,** *52*, 697-703.

Downloaded by UNIV OF GUELPH LIBRARY on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch017

31. Aly, R.; Granot, D.; Mahler-Slasky, Y.; Halpern, N.; Galun, E. *Protein Expression and Purification* **1999,** *16*, 120-124.
32. Boman, H. G.; Hultmark, D. *Annu. Rev. Microbiol.* **1987,** *41*, 103-126.
33. Kanai, A.; Natori, S. *FEBS Lett.* **1989,** *258*, 199-202.
34. Natori, S. *Nippon Rinsho* **1995,** *53*, 1297-1304.
35. Okada, M.; Natori, S. *Biochem. J.* **1985,** *229*, 453-458.
36. Iwai, H.; Nakajima, Y.; Natori, S.; Arata, Y.; Shimada, I. *Eur. J. Biochem.* **1993,** *217*, 639-644.
37. Nakajima, Y.; Qu, X. M.; Natori, S. *J. Biol. Chem.* **1987,** *262*, 1665-1669.
38. Shai, Y. *Biochim. Biophys. Acta* **1999,** *1462*, 55-70.
39. Jaynes, J. M.; Julian, G. R.; Jeffers, G. W.; White, K. L.; Enright, F. M. *Pept. Res.* **1989,** 157-160.
40. Nordeen, R. D.; Sinden, S. L.; Jaynes, J. M.; Owens, L. D. *Plant Sci.* **1992,** *82*, 101-107.
41. Ohshima, M.; Misuhara, I.; Okamoto, M.; Sawano, S.; Nishiyama, K.; Kaku, H.; Natori, S.; Ohashi, Y. *J. Biochem.* **1999,** *125*, 431-435.
42. Mitsuhara, I.; Matsufuru, H.; Ohshima, M.; Kaku, H.; Nakajima, Y.; Murai, N.; Natori, S.; Ohashi, Y. *Mol. Plant Microbe Interact.* **2000,** *13*, 860-868.
43. Sharma, A.; Sharma, R.; Imamura, M.; Yamakawa, M.; Machii, H. *FEBS Letters* **2000,** *484*, 7-11.
44. Mahler-Slasky, Y.; Galili, S.; Perl, A.; Aly, R.; Wolf, S.; Aviv, D.; Galun, E. In *Biology of Root Formation*; Altman, A.; Waisel, Y., Eds.; Plenum Press: N.Y., 1996; pp 187-192.
45. Yamamoto, Y. T.; Taylor, C. G.; Acedo, G. N.; Cheng, C.-L.; Conkling, M. A. *Plant Cell* **1991,** *3*, 371-382.
46. Westwood, J. H.; Yu, X.; Foy, C. L.; Cramer, C. L. *Mol. Plant-Microbe Interact.* **1998,** *11*, 530-536.
47. Cramer, C. L.; Weissenborn, D. L.; Cottingham, C. K.; Denbow, C. J.; Eisenback, J. D.; Radin, D. N.; Yu, X. *J. Nematol.* **1993,** *25*, 507-518.
48. Hamamouch, N.; Westwood, J. H.; Banner, I.; Cramer, C. L.; Gepstein, S.; Aly, R. *Transgen. Res.* **2005,** *(in press).*
49. Okamoto, M.; Mitsuhara, I.; Ohshima, M.; Natori, S.; Ohashi, Y. *Plant Cell Physiol.* **1998,** *39*, 57-63.
50. Mitsuhara, I.; Nakajima, Y.; Natori, S.; Mitsuoka, T.; Ohashi, Y. *Biotechnol. Lett.* **2001,** *23*, 569-573.

Downloaded by UNIV OF GUELPH LIBRARY on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch017

Downloaded by PENNSYLVANIA STATE UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch018

Chapter 18

Brassinosteroids in Induced Resistance and Induction of Tolerances to Abiotic Stress in Plants

Annette Friebe

**Center of Advanced European Studies and Research,
Ludwig-Erhard-Allee 2, 531 Bonn, Germany**

Brassinosteroid phytohormones are known to control important developmental functions in plants, including growth, photomorphogenesis, fertility and seed germination. This paper is focused on the role of these compounds in plant defense against biotic and abiotic stress. Studies of resistance inducing effects and activation of plant tolerances to different abiotic stressors are reviewed. Results of the investigation of signal transduction and regulation of gene expression are discussed with respect to molecular mechanisms of brassinosteroid mediated stress responses in plants. Further scientific research on the plant protecting effects of brassinosteroids may lead to practical applications of these plant growth regulators in agricultural production.

In Natural Products for Pest Management; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

Introduction

The idea of protecting crops by activation of their own plant defense mechanisms has become more and more included in modern integrated pest management. In addition to well-known chemical activators such as 2,6-dichloroisonictinic acid and benzo(1,2,3)thiadiazole-7-carbothiolic acid-s-methyl ester (BTH), a wide variety of natural products have been shown to effectively induce resistance. Not only plant growth-promoting rhizobacteria, but also fungal and bacterial metabolites have been successfully applied as resistance inductors. Plant derived natural products and extracts have also been reported to induce plant resistance to viral, fungal and bacterial pathogens. For instance, resistance-inducing effects have been described for extracts of barley and wheat seeds by Yamada et al. 1990 (*1*) and Hiramoto et al. 1992 (*2*). Leaf extracts of *Hedera helix* (*3,4*) and *Reynoutria sachalinensis*, which is known as the commercial product Milsana (*5,6*) have been used for activation of plant defense against pathogen attacks. Although intensive studies have been undertaken in order to clarify the mode of action of these extracts, little is known about the chemical nature of their active constituents. This paper reviews recent studies of resistance inducing effects of brassinosteroid-containing plant extracts and brassinosteroid compounds. In addition, an overview of protective influences of brassinosteroid compounds under abiotic stress conditions is given.

Induction of disease resistance in plants by brassinosteroid-containing plant extracts and brassinosteroid phytohormones

A seed extract of *Lychnis viscaria* L. as main constituent of a commercial plant growth-promoting agent with respect to resistance inducing effects was investigated by Roth et al. 2000 (*7*). For this study, different plant-pathogen interactions have been investigated. They treated whole plants of tobacco (*N. tabacum* cv. Samsun) and cucumber (*Cucumis sativus* cv. Chinesische Schlange) with aqueous solutions of the seed extract in concentrations of 0.5 up to 10 mg/L. After a time interval of 5 days plants were inoculated with tobacco mosaic virus and *Sphaerotheca fuliginea*, respectively, and disease development was assayed after additional 5 and 10 days. Even for applied concentrations as low as 1 and 2 mg/L, a significantly increased plant resistance to pathogen infections was described by the authors in both cases. In tobacco the mean lesion diameter was reduced of 20 and 36% in comparison to untreated controls. A similar reduction of colony-covered part of leaf surface was found for the disease development of powdery mildew in cucumber. In addition, the spray

Downloaded by PENNSYLVANIA STATE UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch018

treatment of tomato leaflets with solutions of the *L. viscaria* extract resulted in an enhanced resistance to *Botrytis cinerea.*

In tobacco experiments without a time interval between leaf spray treatment and inoculation with TMV, no significant increase of plant resistance was observed, indicating that a time interval was necessary for induction of plant resistance (*7*). In addition, no direct antifungal activity of the investigated plant extract on mycelial growth of *Phytophthora infestans* was observed, even with effector concentrations as high as 1 g/L. Therefore, it was concluded by the authors that application of the plant extract lead to an activation of plant defense mechanisms. This idea was supported by observed stimulations of different plant defense proteins. The enhanced plant resistance was correlated with increased activities of different pathogenesis- related (PR) proteins: chitinase, ß-1,3-glucanase and peroxidase.

Interestingly, Roth et al. showed by SDS-PAGE analysis and time-dependent investigation of the peroxidase activity stimulation for cucumber that leaf spray treatment with the natural product alone was not sufficient to induce the respective PR-proteins. The plant defense reactions were rather triggered by following inoculation of cucumber plants with powdery mildew (*7*). Similar effects have also been described for a variety of inductors of systemic acquired resistance in plants (for review see Conrath et al. 2002 (*8*)). For instance, an activation of the important defense enzyme phenylalanine ammonia-lyase in cowpea after treatment with the synthetic resistance inducer BTH only occurred after following inoculation with pathogen, and was not observed in induced but uninoculated tissues (*9*). In plants, pretreatment with effective inductors primes cells to react more rapidly and efficiently to subsequent pathogenous attack and also abiotic stress conditions. It is hypothesized that an increased presence of cellular signaling components after priming might be responsible for the accelerated and enhanced response to a second stress stimulus (*8*). In comparison to direct upregulation of plant defense genes, the priming of plant cells to potentiated defense in response to stress might have positive effects on ecological costs of induced resistance (*10*).

In preliminary tests Roth (*11*) found that crude fractions of the seed extract exhibited both elicitor and phytohormone activities. Therefore, the resistance-inducing seed extract of *Lychnis viscaria* was subsequently analyzed for brassinosteroid components (*12*). Fractions showing phytohormone activity were isolated from the seed extract by a procedure of solvent partition and subsequent chromatography as shown in Figure 1.

The biological activity of each fraction was monitored by the sensitive rice lamina inclination test (*13*). A biologically active fraction was isolated by preparative HPLC and further analyzed by GC-mass spectrometry after derivatisation with methylboronic acid. As a result, two brassinosteroid compounds have been identified by comparison with respective synthetic

Downloaded by PENNSYLVANIA STATE UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch018

reference compounds and analysis of the fragmentation of the EI mass spectrum. The structures of the identified compounds 24-*epi*-secasterone (**1**) and 24-*epi*-castasterone (**2**) are given in Figure 2. This was the first identification of brassinosteroids in the *Lychnis* genus of Carophyllaceae and the first report of 24-*epi*-secasterone as a natural compound. Interestingly, 24-*epi*-brassinolide was earlier reported also as phytohormone of the 24-epi-series in the Caryophyllaceae *Gypsophila perfoliata* (*14*).

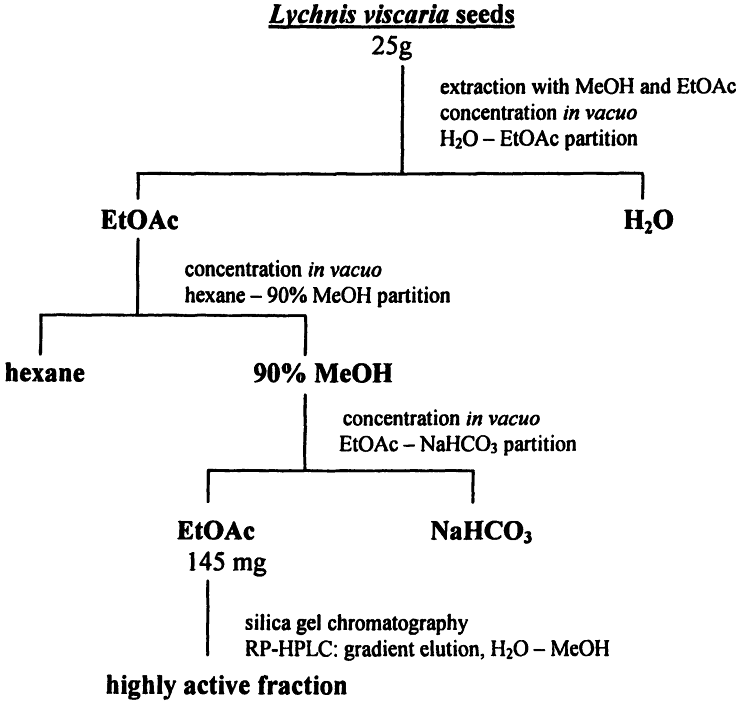
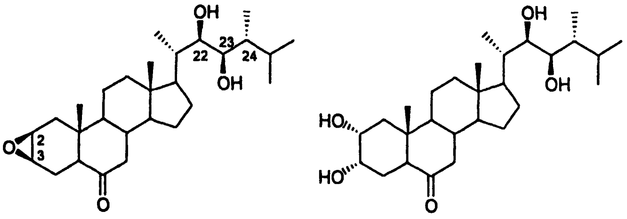

Lychnis viscaria seeds
25g
extraction with MeOH and EtOAc
concentration in vacuo
H_2O – EtOAc partition
EtOAc
H_2O
concentration in vacuo
hexane – 90% MeOH partition
hexane
90% MeOH
concentration in vacuo
EtOAc – $NaHCO_3$ partition
EtOAc
145 mg
$NaHCO_3$
silica gel chromatography
RP-HPLC: gradient elution, H_2O – MeOH
highly active fraction

Figure 1: Isolation procedure for a brassinosteroid containing fraction of a Lychnis viscaria *seed extract as described in refrence* 12.

Experiments which were performed in order to study resistance-inducing effects of brassinosteroids under field conditions have been reviewed by Khripach et al. 1999 (*15*). Protective properties of 24-epi-brassinolide against *Phytophthora* infection have been described for potato. Cultivar-dependent reductions of disease development between 11% and 34% have been observed (*16*). Decreased levels of leaf diseases induced by *Helmintosporium teres* Sacc. have been found in barley plants after spray treatment with 24-*epi*-brassinolide in the tillering phase (*17*). Treatment of cucumber was also shown to result in

Downloaded by PENNSYLVANIA STATE UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch018

enhanced plant resistance to the fungal disease peronosporosis. In this case, the increased plant resistance was correlated with stimulation of peroxidase and polyphenoloxidase enzymes in the leaves of cucumber (*15*).



1 2

Figure 2. Structures of 24-epi-secasterone (1) and 24-epi-castasterone (2) reported as brassinosteroid phytohormones of Lychnis viscaria (12).

More recent studies on resistance inducing effects of brassinolide have been performed using tobacco and rice (*18*). Treatment of tobacco with brassinolide resulted in an increased resistance to the viral pathogen Tobacco mosaic virus, the bacterial pathogen *Pseudomonas syringae* pv. Tabaci as well as the pathogenic fungi *Oidium* sp.. In the monocotyledonous plant rice brassinolide was shown to induce resistance against *Magnaporthe grisea* and *Xanthomonas oryzae* pv. Orycae. It was demonstrated by the authors that levels of free and total salicylic acid were not affected by brassinolide treatment. It was rather shown by Yasuda et al. 2003 (*19*) that brassinolide acts as inductor of disease resistance in *Arabidopsis* against a broad range of pathogens but resistance was not induced in the ethylene insensitive mutant *ein2*. Therefore, the authors hypothesized that ethylene plays a role in brassinosteroid mediated disease resistance.

Effects of brassinosteroids on plant response to abiotic stress

Observations of plant protection against a wide spectrum of environmental stress factors were reported in early documentation of plant promoting activities of brassinosteroid compounds. In field trials yield-increasing effects are often more pronounced under unfavorable growth conditions including non-optimal temperature, light and soil conditions (*20,21,22*). Therefore, an abundance of research has been focused on investigation of enhancement of plant tolerances to abiotic stress. An overview of recent studies is given in Table 1.

Different physiological parameters such as germination, seedling development, plant growth, rooting and fruit setting have been studied in

Downloaded by PENNSYLVANIA STATE UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch018

brassinosteroid treated crop plants in comparison to untreated controls under stress application. Positive effects have been established for brassinosteroid compounds which have been applied in low μM concentrations (e.g. *25,35*) by either seed soaking procedures or leaf spray treatments.

Table I. Studies of Brassinosteroid Effects on Plant Response to Abiotic Stresses.

Ref.	Brassinosteroid	Effect on plant stress response	Abiotic Stress
(*23*)	28-homobrassinolide, 24-epibrassinolide	increase in germination and seedling development of *Sorghum vulgare*	osmotic
(*24*)	synthetic brassinosteroid derivative	stimulation of antioxidant enzymes in *Oryza sativa*	salt
(*25*) (*26*)	28-homobrassinolide, 24-epibrassinolide	improvement of seed germination and increased content of soluble proteins (*Oryza sativa*)	
(*27*)	brassinolide, 24-epibrassinolide, 28-homobrassinolide	improvement of seedling growth of *Arachis hypogaea*	
(*28*)	brassinolide	increase in salt tolerance and yield of *Triticum aestivum*	
(*29*)	brassinolide	increase in tolerance of *Zea mays*	drought
(*30*)	brassinolide	increase in protoplasmic drought tolerance and protein synthesis in *Sporobolus stapfianus* leaf cells	
(*31*)	brassinolide	promotion of cell elongation in young seedlings (*Oryza sativa*)	cold
(*32*)	brassinolide	increase in survival ratio of seedlings (*Oryza sativa*)	
(*33*)	24-epibrassinolide	higher pollen viability of tomato	heat
(*34*)	24-epibrassinolide	protection of translational machinery and heat shock protein synthesis in *Brassica napus* seedlings	
(*35*)	24-epibrassinolide	thermotolerance of *Brassica napus* seedlings	
(*36*)	24-epibrassinolide	induction of thermotolerance of cells of *Bromus inermis*	
(*37*)	brassinolide	promotion of growth and increase in chlorophyll content of mung bean plants	aluminium

Downloaded by PENNSYLVANIA STATE UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch018

24-Epibrassinolide and 28-homobrassinolide have been reported to reduce inhibitory effects of salinity stress on germination and seedling growth of rice. These observations have been correlated with enhanced levels of nucleic acids and soluble proteins (*25,26*). In this context, stimulation of the antioxidant enzymes gluthation reductase, superoxide dismutase and catalase after treatment of rice seedlings with a synthetic brassinosteroid derivative has also been determined (*24*). Interestingly, it has been experimentally shown for sorghum that there is a transient sensitivity to 24-epibrassinolide for a short critical period during salt adaptation. This may be due to changes in sensitivity to growth regulators and interactions with other phytohormones (*38*).

The complex system of antioxidant enzymes is of general importance for plant defense response to oxidative stress caused by different environmental factors. The set of oxygen scavenging defense enzymes in plant cells removes toxic substrates during the increased production of reactive oxygen species under stress. Modulation of antioxydant enzyme activities by brassinosteroid treatment was not only reported for salinity (24) and osmotic stress (*23*), but also for heat stress (*39*).

The influence of 24-epibrassinolide on plant response to heat stress was systematically investigated by Dhaubnadel et al. (*34,35*). Growing of seedlings of tomato and *Brassica napus* in 24-epibrassinolide containing media resulted in an enhanced survival rate after lethal heat treatment. In this study, western blot analysis of heat shock proteins (HSPs) were performed, indicating that higher levels of HSPs in treated seedling were not induced under control temperature (*35*). This is another example for priming of plant by brassinosteroid treatment to potentiate defense responses to a subsequent stress stimulus. Later it was demonstrated that brassinosteroid treatment protects the translational machinery and synthesis of HSPs (*34*). Recently, it was experimentally shown that pollen viability of tomato is also increased by 24-epibrassinolide (*33*).

Downloaded by PENNSYLVANIA STATE UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch018

Molecular aspects of brassinosteroid signal transduction

Results of the above mentioned studies indicate that brassinosteroid phytohormones have the ability to activate plant defense mechanisms to a broad spectrum of biotic and abiotic stresses. Nevertheless, molecular mechanisms of the induction of stress tolerances need to be further clarified. Advances in understanding of the molecular basis of brassinosteroid action in plant cells came from identification of important components of the respective signal cascades (for review see *40,41*). Therefore, some aspects of the signal transduction should be discussed with respect to the possible importance for brassinosteroid-mediated stress response.

On the basis of detailed molecular genetic studies of brassinosteroid-responsive *Arabidopsis* mutants, the brassinosteroid membrane receptor BRI1 and downstream signal components have been identified. The BRI1 gene encodes a leucine rich repeat (LRR) transmembrane receptor-like kinase (RLK) that acts as major brassinosteroid receptor. Later a second LRR-RLK BAK1 was identified which is involved in mediation of brassinosteroid perception by interaction with BRI1. Interestingly, it was later shown by Montoya et al. 2002 (*42*) that the tomato ortholog tBRI1 also acts as receptor for the peptide hormone systemin. Systemin mediates stress response in tomato partly through induction of jasmonic acid. Nevertheless, the reason for this dual function of the receptor in this plant species still remains unclear.

Experimental studies of Sharma et al. 2001 (*43*) revealed the activation of a membrane bound mitogen-activated protein kinase (MAPK) in rice by exogenous application of brassinolide. MAPK cascades are among the major signal pathways involved in regulation of plant stress response. Besides responding to diverse environmental stress stimuli and pathogen infection, MAPKs are also known to respond to different plant hormones such as jasmonic acid, ethylene or salicylic acid and important inductors of plant resistance like BTH (*44*).

Different experimental techniques have been applied in order to study brassinosteroid regulation of gene expression. Besides classical methods, cDNA and oligonucleotide microarrays have been used to identify genes, which are upregulated or downregulated after brassinosteroid treatment. Some of the results gave further support for the multiple roles of brassinosteroids in mediation of plant response to environmental stress. Results of a DNA chip-based expression profile analysis showed that 24-epibrassinolide also regulates components of the important phosphatidylinositol pathway (*45*).

Conclusions and future prospects

A number of experimental studies under laboratory and field conditions have revealed an activation of plant defense mechanisms to biotic and abiotic stress by brassinosteroid compounds. Protective influences are thought to contribute to yield increasing effects of brassinosteroid phytohormones, synthetic brassinosteroid derivatives and brassinosteroid–containing natural products. Recent molecular genetic studies have greatly improved the knowledge about signal transduction and regulation of gene expression. The ability of brassinosteroids to induce tolerance in plants to a broad spectrum of stress factors seems to result largely from interactions with other phytohormones. Further investigation on the molecular basis of brassinosteroid-mediated stress response and interactions with environmental stimuli will have a great impact on future practical application of these plant-promoting substances in crop production.

Downloaded by PENNSYLVANIA STATE UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch018

References

1. Yamada, T.; Hiramoto, T.; Tobimatsu, R.; Shiraishi, T.; Oku, H. *J. Phytopathology* **1990,** *128*, 89-98.
2. Hiramoto, T.; Tobimatsu, R.; Shiraishi, T., Yamada, T.; Ichinose, Y.; Oku, H. *J Phytophatology* **1992,** *135*, 167-176.
3. Rohner, E.; Carabet, A.; Buchenauer, H. *J. Plant Diseases Protection* **2004,** *111*, 83-95.
4. Mende, A.; Mosch, J.; Zeller, W. *J. Plant Diseases Protection* **1994,** *101*, 141-147.
5. Konstantinidou-Doltinis, S.; Schmitt, A. *Crop Protection* **1998,** *8*, 649-656
6. Fofana, B.; McNally, D. J.; Labbe, C.; Boulanger, R.; Benhamou, N.; Seguin, A.; Belanger, R. R. *Physiol. Mol. Plant Pathol.* **2002,** *61*, 121-132.
7. Roth, U.; Friebe, A.; Schnabl, H. *Z. Naturforsch.* **2000,** *55c*, 552-559.
8. Conrath, U.; Pieterse, C. M. J.; Mauch-Mani, B. *Trends Plant Sci.* **2002,** *7*, 210-216.
9. Akinwunmi, O. L.-D.; Lucas, J. A. *Physiol. Mol. Plant Pathol.* **2001,** 199-208.
10. Heil, M. *Curr. Opin. Plant Biol.* **2002,** *5*, 345-350.
11. Roth, U. Ph.D . thesis, University of Bonn, Bonn, GE, 2000
12. Friebe, A.; Volz, A.; Schmidt, J.; Voigt, B.; Adam, G.; Schnabl., H. *Phytochemistry* **1999,** *52*, 1607-1610.
13. Arima, M.; Yokota, T.; Takahashi, N. *Phytochemistry*, **1984,** *23*, 1587 – 1591.
14. Schmidt, J.; Böhme, F.; Adam, G. *Z. Naturforsch.* **1996,** *51c*, 897-899.
15. *Brassinosteroids. A New Class of Phytohormones;* Khripach, V. A.; Zhabinskii, V. N.; de Groot, V. E., Eds.; Academic Press: San Diego, CA, 1999, pp 277-285.
16. Khripach, V. A.; Zhabinskii, V. N.; Malevannaya, N. N. *Proc. Plant Growth Regul. Soc. Am.* **1997,** 101-106.
17. Volynets, A. P.; Pschenichnaya, L. A.; Manzhelesova, N. E.; Morozik, G. V.; Khripach, V. A. *Proc. Plant Growth Reg. Soc. Am.* **1997,** *24*, 133-137.
18. Nakashita, H.; Yasuda, M.; Nitta, T.; Asami, T.; Fujioka, S.; Arai, Y.; Sekimata, K.; Takatsuto, I.; Yoshida, S. *Plant J.* **2003,** *33*, 887-898.
19. Yasuda, M., Nakashita, H., Asami, T.; Yoshida, S. *Plant Cell Physiol.* **2003,** *44* supplement, 178.
20. Pigorovskaya, G. V.; Bogdevitch, I. M.; Naumova, G. V.; Khripach, V. A.; Azizbekyan, S. G.; Krul, L. P. *Proc. Plant Growth Regul. Soc. Am.* **1996,** *23*, 146-151.
21. Kamuro, Y.; Takatsuto, S.; Watanabe, T.; Noguchi, T.; Kuriyama, H.; Suganuma, H. *Proc. Plant Growth Regul. Soc. Am.* **1997,** 111-116.

Downloaded by PENNSYLVANIA STATE UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch018

22. *Brassinosteroids. A New Class of Phytohormones;* Khripach, V. A.; Zhabinskii, V. N.; de Groot, V. E., Eds.; Academic Press: San Diego, CA, 1999, pp 325-346.
23. Vardhini, B. V.; Rao, S. S. R. *Plant Growth Regul.* **2003,** *41,* 25-31.
24. Nunez, M.; Mazzafera, P.; Mazorra, L. M.; Siqueira, W. J.; Zullo, M. A. T. *Biol. Plantarum,* **2003,** 47, 67-70.
25. Anuradha, S.; Rao, S. S. R. *Plant Growth Regul.* **2001,** *33,* 151-153.
26. Anuradha, S.; Rao, S. S. R. *Plant Growth Regul.* **2003,** *40,* 29-32.
27. Vardhini, B. V.; Rao, S. S. R. *Indian J. Plant Physiol.* **1997,** *2,* 156-157.
28. Hathout, T. A. *Egypt. J. Physiol. Sci.* **1996,** *20,* 127-152.
29. Li, L.; Van Staden, J.; Jager, A. K. *Plant Growth Regul.* **1998,** *25,* 81-87.
30. Ghasempour, H. R.; Anderson, E. M.; Gianello, R. D.; Gaff, D. F. *Plant Growth Reg.* **1998,** *24,* 179-183.
31. Fujii, S.; Saka, H. *Plant Prod. Sci.* **2001,** *4,* 210-214.
32. Hotta, Y.; Tanaka, T.; Luo, B. S.; Takeuchi, Y.; Konnai, M. *J. Pesticide Sci.* **1998,** *23,* 29-33.
33. Singh, I.; Shono, M. *Indian J. Exp. Biol.* **2003,** *41,* 174-176.
34. Dhaubhadel, S.; Browning, K. S.; Gallie, D. R.; Krishna, P. *Plant J.* **2002,** *29,* 681-91.
35. Dhaubhadel, S.; Chaudhary, S.; Dobinson, K. F.; Krishna, P. *Plant Mol. Biol.* **1999,** *40,* 333-342.
36. Wilen, R. W.; Sacco, M.; Gusta, L. V.; Krishna, P. *Physiol. Plantarum* **1995,** *95,* 195-202.
37. Abdullahi, B. A.; Gu, X.-G.; Gan, Q.-L.; Yang, Y.-H. *J. Plant Nutrition* **2003,** *26,* 1725-1734.
38. Amzallag, N. G. *Isreal J. Plant Sci.* **2004,** *52,* 1-8.
39. Mazorra, L. M.; Nunez, M.; Hechavarria, M.; Coll, F.; Sanchez-Blanco, M. J. *Biologia Plantarum* **2002,** *45,* 593-596.
40. Clouse, S. D. *Mol. Cell* **2002,** *10,* 973-982.
41. Li, J.*Current Opinion Plant Biol.* 2003, *6,* 494-499.
42. Montoya, T.; Nomura, T.; Farrar, K.; Kaneta, T.; Yokota, T.; Bishop, G. J. *Plant Cell* **2002,** *14,* 3163-76.
43. Sharma, A.; Matsuoka, M.; Tanaka, H.; Komatsu, S. *FEBS Lett.* **2001,** *507,* 346-350.
44. Agrawal, G. K.; Iwahashi, H.; Rakwal, R. *Biochem. Biophys. Res. Commun.* **2003,** *302,* 171-80.
45. Lin, W. H.; Ye, R.; Ma, H.; Xu, Z. H. ; Xue, H. W. *Cell Res.* **2004,** *14,* 34-45.

Downloaded by PENNSYLVANIA STATE UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch018

Chapter 19

Actinonin-Induced Inhibition of Plant Peptide Deformylase: A Paradigm for the Design of Novel Broad-Spectrum Herbicides

Cai-Xia Hou and Mark Williams*

Department of Horticulture, University of Kentucky, N322D Agriculture Science Center, Lexington, KY 40546

Downloaded by YORK UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch019

Peptide deformylase, which catalyzes the removal of N-formyl groups from the initiating N-formyl-methionine of nascent polypeptides, has recently been characterized from several plants, including rice, tomato and *Arabidopsis thaliana*. The two *Arabidopsis thaliana* DEF genes, *AtDEF1* and *AtDEF2*, encode enzymes which are functionally active both *in vitro* and *in vivo* and are catalytically inactivated by the naturally-occurring peptide deformylase inhibitor actinonin, a product of a soil-borne actinomycete. Actinonin has profound herbicidal effects when applied to many plant species both pre- and post-emergence. Transgenic tobacco plants were engineered to over-express each of the *At*DEF proteins. These plants were completely resistant to the herbicidal effects of actinonin. This data provides the first unequivocal evidence that the lethality of actinonin to plants *in vivo* is strictly a consequence of the inhibition of peptide deformylase activity. This work also confirms that peptide deformylase is a valid target for both the development of novel broad-spectrum herbicides, and the engineering of herbicide selectivity in plants without the use of foreign genes.

In Natural Products for Pest Management; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

Downloaded by YORK UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch019

Almost all protein synthesis is initiated with a methionine residue. In prokaryotes, as well as in the organelles of eukaryotes, this methionine is modified by an N-formyl group. However, the majority of mature proteins (*1*) in these organisms do not contain a methionine residue at the N-terminus as a consequence of sequential and specific co-translational processing by two enzymes, peptide deformylase (DEF) and methionine aminopeptidase (MAP) (*2, 3*). Protein processing by DEF and MAP are essential processes and their significance is underscored by the lethality of E. coli null mutants (*4, 5*) and antibacterial activity of DEF inhibitors (*6-10*).

The exact mechanism by which DEF activity is required for cell viability is unclear; yet there are examples where the N-terminal residue of a protein is catalytically or structurally active (*11-13*) or influential as a determinant in protein stability (*14*). However, a generality which explains the absolute necessity of DEF activity is lacking. The essentiality of DEF and its initially presumed restriction to prokaryotic organisms has made it the subject of many research endeavors, as an ideal target for the development of broad-spectrum antibiotics (*15-20*).

There have been several NMR solution structures (*21-23*) and high-resolution x-ray crystallography structures (*24-27*) published for bacterial DEF. Due to extraordinary lability ($t_{1/2}$ ~ 1 min at room temperature; *28*), the enzyme was originally characterized only after the gene was cloned (*4*) and the protein over-expressed (*29*). Since then, a plethora of biochemical and enzymological data has become available on this enzyme including two convenient enzymatically-coupled assays (*30, 31*).

In this chapter we describe research identifying and characterizing two plant peptide deformylase genes from *Arabidopsis thaliana.* Enzymes encoded by these genes are inactivated by the naturally occurring peptide deformylase inhibitor actinonin. The functional significance of inhibiting these enzymes and the production of transgenic plants that have engineered resistance to actinonin is examined and underscores the potential for using peptide deformylase as a novel broad-spectrum herbicidal target.

Initial Characterization of Plant Peptide Deformylase

Two putative peptide deformylase enzymes (*At*DEF1 and *At*DEF2) were initially identified from the Arabidposis Genome Initiative by searching the genomic sequence database for homologs of *E. coli* peptide deformylase. The deduced translation products (*32*) each contain an N-terminal chloroplast targeting sequence as predicted by ChloroP v1.1 (*33*). Comparison of the deduced translational sequences indicate that there is only 17% identity and 44% similarity between the *At*DEFs and the *E. coli* protein (*32*). This low degree of homology is typical of that observed between other eubacterial peptide deformylases, with the highest sequence conservation occurring at the three active site regions (17).

Since both of the *At*DEF enzymes are nuclear encoded and presumably function in the chloroplast, it was critical to examine whether the predicted chloroplast targeting sequences were sufficient to direct these enzymes to the organelle. *In vitro* uptake and processing experiments using radiolabelled full-length AtDEF1 confirmed that the predicted chloroplast-targeting sequence was indeed sufficient to direct import into isolated intact pea chloroplasts (*32*). Transient expression studies using *At*DEF1 and 2 proteins fused with green fluorescent protein also confirmed that both proteins were capable of being imported into chloroplasts *in vivo* (*34*). Additionally, antibodies against *At*DEF1 and 2 specifically detected both proteins in Arabidopsis chloroplast stromal extracts, further confirming that the enzymes are activily imported into the organelles *in vivo* (*32*).

Downloaded by YORK UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch019

To confirm that the *At*DEFs had actual deformylase activity, the processed forms were expressed in *E. coli* as fusions with C-terminal hexahistidyl tags, which facilitated the affinity purification of the proteins. Purified *At*DEF 1 and 2 were then assayed with two different spectrophotometric peptide deformylase methods (*32*). One of the methods utilizes N-formyl-Met-Leu-ρ-nitroanilide as a N-terminal substrate mimmick in an enzyme-linked assay containing the substrate, the putative deformylase, and an aminopeptidase from *Aeromonas proteolytica* (*30*). Removal of the formyl group by peptide deformylase is required for the cleavage of the Met and Leu residues by the aminopeptidase. Once this occurs, the ρ-nitroanilide group is released and can be specrophotometrically detected (Figure 1). Both of the putative *At*DEFs were confirmed to have peptide deformylase activity when assayed in this way and kinetic parameters were were determined (*32, 35*). Attempts to detect activity in isolated Arabidopsis chloroplasts and leaf extracts have not been successful, possibly due to the low abundance and high lability of the enzyme.

Effects of Actinonin on Plant Peptide Deformylase

The most potent naturally-occurring inhibitor of peptide deformylase discovered thus far is the compound actinonin, which is produced by a soil-borne actinomycete (*7, 10*). The structure of actinonin has been determined (Figure 2) and resembles a pseudopeptide with an N-terminal Met analog. This compound is thought to act as a "molecular mimmick" of *in vivo* peptide deformylase substrates, and is therefore capable of binding in the active site of the enzyme. Once this occurs, the hydroxamate group of actinonin is thought to chelate the active site metal ion, thus inhibiting enzyme activity (*7*).

Downloaded by YORK UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch019

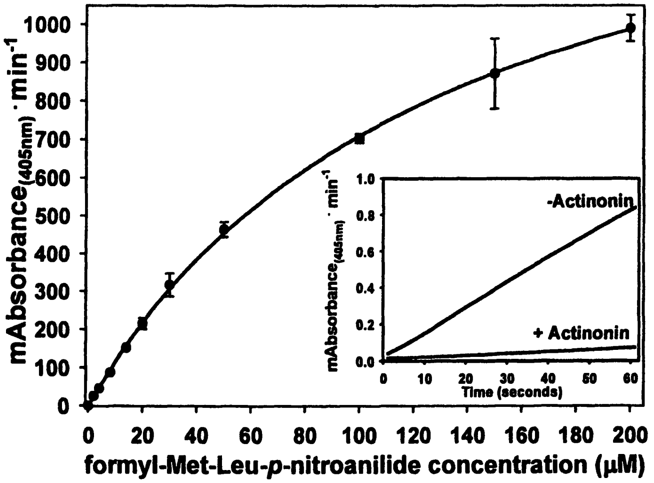


Figure 1. Kinetic analysis of AtDEF2 activity. Data was obtained using increasing N-formyl-Met-Leu-p-nitroanilide concentrations as indicated, 7.5 μg of purified AtDEF2 protein , and 1.0 unit of Aeromonas proteolytica aminopeptidase. N=5. Error bars = SEM. The inset panel shows the inhibition of AtDEF2 by actinonin. For the actinonin assay, 7.5 ug of AtDEF2 was preincubated for 3 minutes in the absence or presence of 300 nM actinonin prior to initiating the assay with 200 μM N-formyl-Met-Leu-p-nitroanilide.

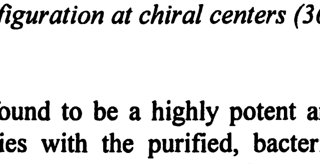


Figure 2. Structure of actinonin. R *and* S *indicate configuration at chiral centers (36).*

Actinonin has been found to be a highly potent and selective inhibitor of bacterial DEFs (*7*). Studies with the purified, bacterially-expressed, *At*DEFs showed that plant DEFs are also extremely sensitive to actinonin inhibition to an extent similar to that observed in bacteria (Figure 1, inset; *32, 35*). Actinonin has been demonstrated to have pre-emergence herbicidal activity by inhibiting seed germination (*32, 37*), and inducing an albino phenotype in germinated seedlings (*32, 35, 37*). Additionally, actinonin also has been found to have post-emergence herbicidal activity and results in a rapid bleaching of developing leaves that ultimately leads to tissue necrosis after topical application (Figure 3; *32, 37*).

Downloaded by YORK UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch019

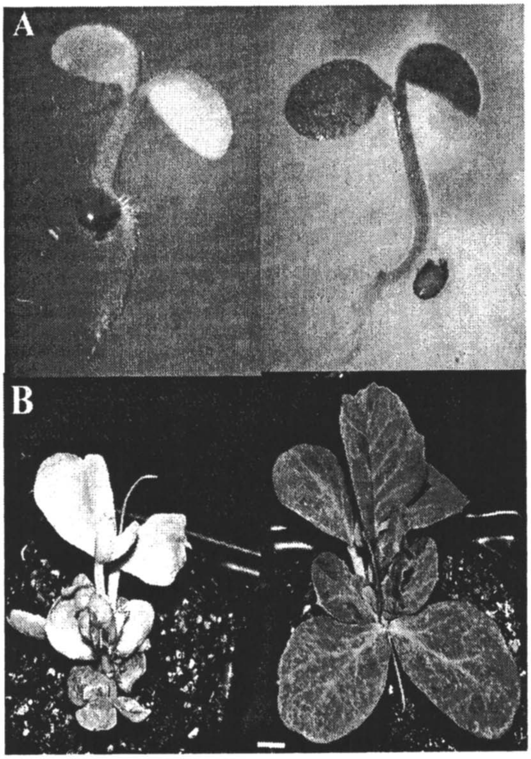


Figure 3. Actinonin treatment of tobacco (A) and pea (B). (A) Tobacco seeds (cv. Kentucky 14) were imbibed and seedlings cultured at room temperature with constant light (50 µmole.m-2.s-1) in Murashige and Skoog basal salts with 0.2% phytagel for 7 days in the absence (right) or presence (left) of 0.65 mM actinonin. (B) Approximately 100-200 µl 6.5 mM actinonin was applied per pea plant in 0.1% Tween-20 (left). Control plants(right) were treated with 0.1% Tween-20. Means (n=12) of fresh (dry) weights were 0.72 g (0.11 g) and 0.43 g (0.063 g) for Tween-20-treated and actinonin-treated pea plants (16 day-old), respectively. (See page 1 of color inserts.)

These observations all support the hypothesis that chloroplast-localized DEF is indispensable for plant growth and development (*38, 32, 35*) and that actinonin is an ideal tool for determining the functional significance of plant DEFs as well as providing an excellent basis for designing a new class of broad-spectrum herbicides.

Functional Consequences of Peptide Deformylase Inhibition

Inhibition of DEF would theoretically result in an accumulation of proteins with N-formylated methionine residues, and this hypothesis was confirmed recently in prokaryotes when actinonin treatment resulted in an accumulation of proteins with N-formylated termini (*39, 40*). Although similar consequences would be expected in eukaryotic organisms, there has been a lack of direct identification of the results of DEF inhibition. Inhibition of chloroplast-localized DEFs could potentially compromise co-translational protein processing and thus, protein function in all plant plastids (*32*). Studies using polypeptide mimics of in vivo DEF substrates in in vitro DEF reactions revealed that the D1 protein, which is one of the core proteins in photosystem II (PSII) is a preferred substrate of DEF (*41*). Recent results have demonstrated that inhibition of DEF by actinonin in tobacco (*Nicotiana tobacum)* results in a reduction of PSII activity manifested as a significant decrease in chlorophyll fluorescence as measured by Fv/Fm. This reduction occurs rapidly and is observable after just 24 hours of actinonin treatment. Nascent D1 protein synthesis and assembly into PSII monomers was also found to be reduced, eventually leading to PSII disassembly, a concomminant loss in photosynthesis, and ultimately cell death (Figure 4; *37*). These results were similar to those observed in *Chlamydomonas* in that actinonin treatment also resulted in a degradation of newly synthesized PSII proteins (42). In *Chlamydomas* however, PSII degradation appears to be a direct consequence of D2 instabilty and degradation. Thus, the herbicidal action of DEF inhibitors appears to be the processing and assembly of PSII subunit polypeptides, leading to severe inhibition of photosynthesis. These results confirm that N-terminal deformylation is an essential step in the accumulation and assembly of PSII subunit polypeptides in chloroplasts.

Engineered Resistance to the Actinonin

The use of actinonin as a DEF inhibitor has been instrumental in the elucidation of many fundamental aspects of plant DEFs. The ensuing leaf necrosis and eventual plant death resulting from this treatment has demonstrated the functional significance of DEF as well as providing evidence for the mode of

Downloaded by YORK UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch019

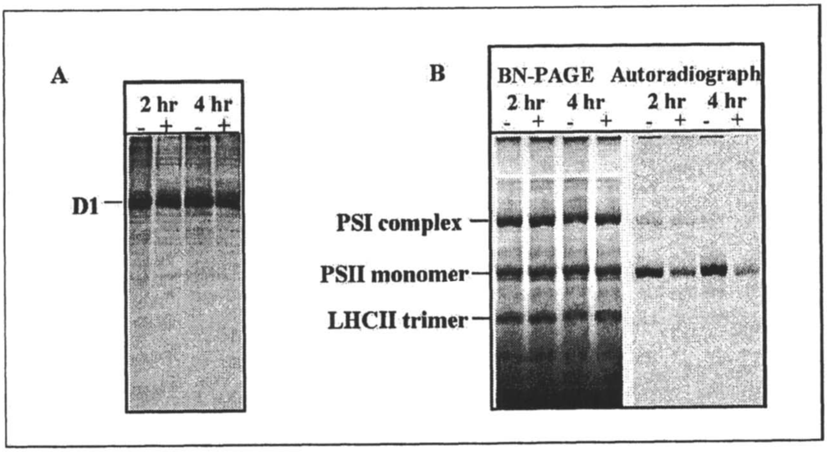


Figure 4. The effects of actinonin on D1 protein synthesis in leaf discs from tobacco seedlings. Leaf discs from 35-d-old tobacco plants were pretreated with 1 mM actinonin in 0.05% (v/v) Tween 20 (+) or 0.05% (v/v) Tween 20 (-) for 2 or 4 h. 35S-methionine (7 μCi/mL) was then added to initiate a 2-h in vivo labeling of protein synthesis. A. Phosphorimage of thylakoid membrane proteins (3 μg chlorophyll/well) separated by SDS-PAGE .The D1 band was identified by Western analysis with a D1-specific antibod. B. Blue-native PAGE profile of thylakoid membrane protein complexes (left panel, 15 μg chlorophyll/well) and its corresponding phosphorimage (right panel). This experiment was repeated four times. Label incorporation was quantified for each band as described in (37). Complexes are named on the left side.

Downloaded by YORK UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch019

action of DEF inhibitors. The ability of actinonin to effectively kill a wide range of plants, including many difficult to control weed species, has confirmed that herbicides targeting DEFs will truly have broad-spectrum activity.

Recent research with peptide deformylase has focused on the construction of transgenic tobacco plants with engineered resistance to the herbicidal effects of actinonin. cDNAs from Arabidopsis *AtDEF* genes were cloned into a strong constituitive promoter (MMV FLt) and these constructs were transformed into tobacco plants using *Agrobacterium*-mediated transformation. High level expression of *At*DEF1 and 2 was confirmed in leaf extracts from transgenic tobacco using Western blot analysis (Figure 5). The results demonstrate that wild-type tobacco or tobacco transformed with an empty vector do not show immunological signals for *At*DEF1 or 2. However, several plants selected from two independent lines of *At*DEF1 and 2 over-expressors show high-amounts of AtDEF1 and 2 proteins, respectively. Preliminary quantification by ELISA analysis suggest that *At*DEF1 and 2 expression is increased as much as 50-fold above normal in the transgenic lines. These plants were also subjected to lethal amounts of actinonin and evaluated for subsequent growth and development. The results dramatically demonstrate that over-expression of either AtDEF1 or 2 is sufficient to convey complete resistance to actinonin in germinating seedlings (Figure 6). Because actinonin is capable of inhibiting other enzymes in addition to DEF, and individual gene knockouts of either DEF1 or 2 alone in Arabidopsis are not lethal, these observations confirm that: 1) DEF activity is essential for plant survival; and 2) that the *in vivo* molecular target of actinonin is DEF.

Downloaded by YORK UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch019

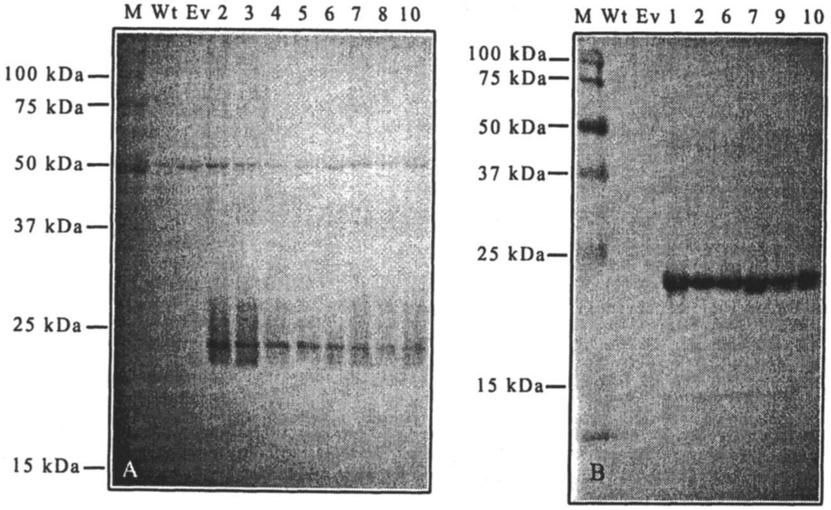


Figure 5. Western analysis of leaf extracts from tobacco plants with engineered over-expression of Arabidopsis Atdef genes. Soluble leaf proteins were extracted from transgenic tobacco lines, harboring At def1 (panel B) and At def2 (panel A) genes, respectively, and 50 μg of protein per lane was fractionated by SDS-PAGE. Proteins were transferred to Immobilon-P membranes and incubated with Atdef1 (panel B) or Atdef2 (panel A) specific antibodies and visualized at ≈23 kDa using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Wt= wild type Arabidopsis, Ev=Arabidopsis transformed with an empty expression vector containing no insert. M=protein molecular weight standards.

Downloaded by YORK UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch019

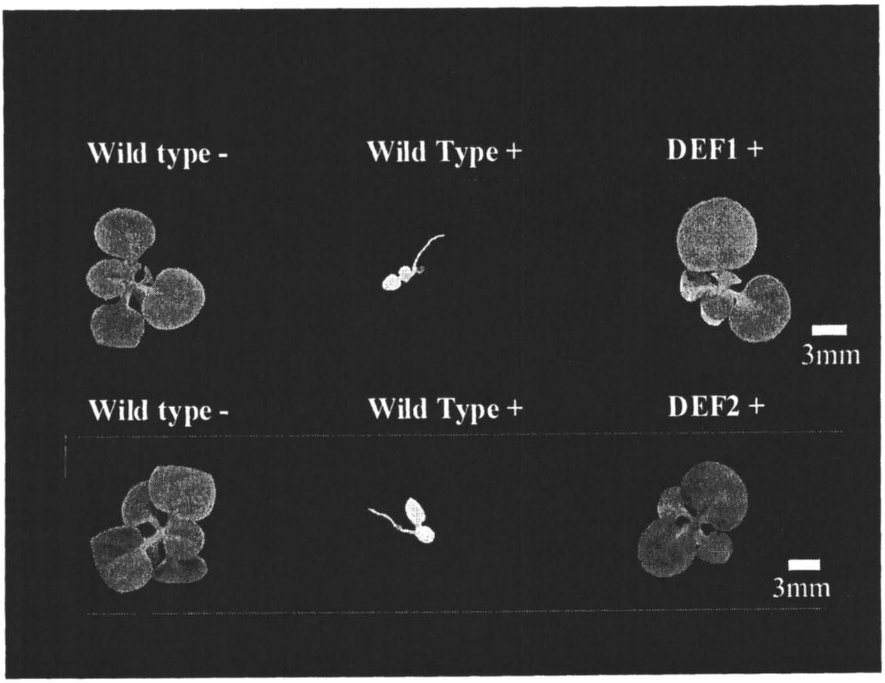


Figure 6. Actinonin treatment of Atdef over-expressing tobacco plants. Tobacco seeds of wild type orDEF1 or DEF2over-expressing plants were germinated in a nutrient- containing agarose medium with (+) or without (-) 1.2 mM actinonin. Photos were taken 42 days after germination. (See page 2 of color inserts.)

Acknowledgements

This work was supported by a Kentucky Tobacco Development Research Center Grant (no. 5-41176) and a National Science Foundation Award (no. MCB-0240165).

References

1. Horikoshi, K.; Doi, R. H. *J. Biol. Chem.* **1968,** *243,* 2381-2384.
2. Takeda, M.; Webster, R. E. *Proc. Natl. Acad. Sci. USA.* **1968,** *60,* 1487-1494.
3. Solbaiti, J.; Chapman-Smith, A.; Miller, J.L., Miller, C. G.; Cronan Jr., J. E. *J. Mol. Biol.* **1999,** *290,* 607-614.
4. Mazel, D.; Pochet, S.; Marliere, P. *EMBO J.* **1994,** *13,* 914-923.
5. Chang, S. Y.; McGary, E. C.; Chang, S. *J. Bacteriology.* **1989,** *171,* 4071-4072.
6. Apfel, C.; Banner, D.W.; Bur, D.; Dietz, M.; Hirata, T.; Hubschwerlen, C.; Locher, H.; Page, M. G.; Pirson, W.; Rosse, G.; Specklin, J. L. *J. Med. Chem.* **2000,** *43,* 2324-2331.
7. Chen, D. Z.; Patel, D.V.; Hackbarth, C. J.; Wang, W.; Dreyer, G.; Young, D. C.; Margolis, P. S.; Wu, C.; Ni, Z. J.; Trias, J.; White, R. J.; Yuan, Z. Y. *Biochem.* **2000,** *39,* 1256-1262.
8. Huntington, K. M.; Yi, T.; Wei, Y.; Pei, D. *Biochem.* **2000,** *39,* 4543-4551.
9. Jayasekera, M. M.; Kendall, A.; Shammas, R.; Dermyer, M.; Tomala, M.; Shapiro, M. A.; Holler, T. P. *Arch. Biochem. Biophys.* **2000,** *381,* 313-316.
10. Margolis, P. S.; Hackbarth, C. J.; Young, D. C.; Wang, W.; Chen, D.; Yuan, Z.; White, R.; Trias, J. *Antimicrob. Agents. Chemother.* **2000,** *44,* 1825-1831.
11. Martinez, S. E.; Huang, D.; Szczepaniak, A.; Cramer, W.A.; Smith, J. L. *Structure.* **1994,** *2,* 95-105.
12. Lowe, J.; Stock, D.; Jap, B.; Zwickl, P.; Baumeister, W.; Huber, R. *Science* **1995,** *268,* 533-539.
13. Seemuller, E.; Lupas, A.; Stock, D.; Lowe, J.; Huber, R.; Baumeister, W. *Science.* **1995,** *268,* 579-582.
14. Tobias, J. W.; Shrader, T. E.; Rocap, G.; Varshavsky, A. *Science.* **1991,** *254,* 1374-1377.
15. Hu, Y. J.; Rajagopalan, P. T.; Pei, D. *Bioorg. Med. Chem. Lett.* **1998,** *8,* 2479-2482.
16. Durand, D. J.; Gordon, G. B.; O'Connell, J. F.; Grant, S. K. *Arch. Biochem. Biophys.* **1999,** *367,* 297-302.

Downloaded by YORK UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch019

17. Hao, B.; Gong, W.; Rajagopalan, P. T.; Zhou, Y.; Pei, D.; Chan, M. K. *Biochem.* **1999**, *38*, 4712-4719.
18. Meinnel, T.; Patiny, L.; Ragusa, S.; Blanquet, S. *Biochem.* **1999**, *38*, 4287-4295.
19. Gordon Green, B. G.; Toney, J. H.; Kozarich, J. W.; Grant, S. K. *Arch. Biochem. Biophys.* **2000**, *375*, 355-358.
20. Wei, Y.; Yi, T.; Huntington, K. M.; Chaudhury, C.; Pei, D. *J. Comb. Chem.* **2000**, *2*, 650-657.
21. Dardel, F.; Ragusa, S.; Lazennec, C.; Blanquet, S.; Meinnel, T. *J. Mol. Biol.* **1998**, *280*, 501-513.
22. Leiting, B.; Marsilio, F.; O'Connell, J. F. *Anal. Biochem.* **1998**, 265, 351-355.
23. O'Connell, J. F.; Pryor, K. A. D.; Grant, S. K.; Leiting, B. *J. Biomolecular NMR.* **1999**, *13*, 311-324.
24. Chan, M. K.; Gong, W.; Rajagopalan, P. T. R.; Hao, B.; Tsai, C. M.; Pei, D. *Biochem.* **1997**, *36*, 13904-13909.
25. Becker, A.; Schlicting, I.; Kabsch, W.; Schultz, S.; Wagner, A.F.V. *J. Biol. Chem.* **1998**, *273*, 11413-11416.
26. Becker, A.; Schlicting, I.; Kabsch, W.; Groche, D.; Schultz, S.; Wagner, A. F. V. *Nat. Struct. Biol.* **1998**, *5*, 1053-1058.
27. Groche, D.; Becker, A.; Schlichting, I.; Kabsch, W.; Schultz, S.; Wagner, A. F. V. *Biochem. Biophys. Res. Commum.* **1998**, *246*, 342-346.
28. Rajagopalan, P. T.; Pei, D. *J. Biol. Chem.* **1998**, *273*, 22305-22310.
29. Rajagopalan, P.T.R.; Datta, A.; Pei, D. *Biochem.* **1997**, *36*, 13910-13918.
30. Wei, Y.; Pei, D. *Anal. Biochem.* **1997**, *250*, 29-34.
31. Lazennec, C.; Meinnel, T. *Anal. Biochem.* **1997**, 244, 180-182.
32. Dirk, L. M. A.; Williams, M. A.; Houtz, R. L. *Plant Phys.* **2001**, *127*, 97-107.
33. Emanuelsson, O.; Nielson, H.; von Heijne, G. *Protein Sci.* **1999**, *8*, 978-984.
34. Dinkins, R. D.; Conn, H. M.; Dirk, L. M. A.; Williams, M. A.; Houtz, R. L. *Plant Science.* **2003**, *165*, 751-758.
35. Serero, A. C.; Giglione, C.; Meinnel, T. *Journal of Mol. Bio.* **2001**, 314, 695–708.
36. Umezawa, H.; Aoyagi, T.; Tanaka, T.; Suda, H.; Okuyama, A.; Naganawa, H.; Hamada, M.; Takeuchi, T. *J. Antibiotics.* **1985**, *38*, 1629-1630.
37. Hou, C.; Dirk, L. M. A.; Williams, M. A. *Am. J. Bot.* **2004**, *91*, 1304-1311.
38. Hanson, A. D.; Gage, D. A.; Shachar-Hill, Y. *Trends in Plant Science.* **2000**, *5*, 206–213.
39. Solbiati, J.; Chapman-Smith, A.; Cronan Jr., J. E. *J. Bio.l Chem.* **2002**, *277*, 21604–21609.

Downloaded by YORK UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch019

40. Bandow, J. E.; Becher, D.; Büttner, K.; Hochgräfe, F.; Freiberg, C.; Brötz, H.; Hecker, M. *Proteomics*. **2003**, *3*, 299–306.

41. Dirk, L. M. A.; Williams, M. A.; Houtz, R. L. *Arch. Biochem. Biophys.* **2003**, *406*, 135–141.

42. Giglione, C.; Vallon, O.; Meinnel, T. *EMBO Journal.* **2003**, 22, 13-23.

Downloaded by YORK UNIV on July 7, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch019

Downloaded by UNIV OF GUELPH LIBRARY on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch020

Chapter 20

Global Gene Expression Approaches to Mode-of-Action Studies with Natural Product-Based Pesticides

Isabelle A. Kagan[1,2], Albrecht Michel[1,3], Brian E. Scheffler[1,4],
Anja Prause[1], Scott R. Baerson[1], and Stephen O. Duke[1]

[1]Natural Products Utilization Research Unit, Agricultural Research
Service, U.S. Department of Agriculture, P.O. Box 8048,
University, MS 38677
[2]Current address: FAPRU, Agricultural Research Service, U.S. Department
of Agriculture, N220 Agricultural Sciences North, Lexington, KY 40546
[3]Current address: Herbicide Profiling, Biology, Syngenta Crop Protection
AG, Werk Stein (WST–149.E.62), CH–4332 Stein, Switzerland
[4]Current address: CGRU, Agricultural Research Service, U.S. Department
of Agriculture, MSA Genomics Laboratory, 141 Experiment Station Road,
Stoneville, MS 38776–0038

Natural products represent a vast repository of potential pesticides, some with novel molecular target sites. Whole-genome DNA microarrays seem a promising tool for identifying novel target sites, as microarrays enable monitoring the effects of a toxicant on the expression of every gene of an organism, providing clues about the targeted pathway or process. Examples of microarray studies of fungicide effects on *Saccharomyces cerevisiae* (baker's yeast), and of the effects of a natural herbicide on the dicot *Arabidopsis thaliana*, are presented. The results support a role for microarray studies in identifying molecular target sites and understanding the responses of plants to xenobiotics.

In Natural Products for Pest Management; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

Introduction

Natural products, well known as a source of novel pharmaceuticals, are also a promising source of novel agricultural pesticides, which are constantly needed as the current ones select for resistant pests (*1*). One approach to overcome resistance is to introduce new pesticides with molecular targets different from those targeted by the previously-used pesticides (*2*). Determining the molecular target of a pesticide can be a slow process of assaying for activities or products of candidate enzymes, and identifying all affected physiological processes is difficult.

The advent of DNA microarrays may streamline identifying candidates for the mode of action of a pesticide, as well as providing more information on changes occurring in the target organism. A microarray is a solid matrix such as a glass slide or a silicon chip onto which known DNA sequences (cDNA's or synthetic oligonucleotides) are deposited by a robotic spotter or synthesized by photolithography (*3*,*4*). In the microarray system using glass slides, RNA or cDNA, coupled to fluorescent dyes, is hybridized to the DNA on the slide, and fluorescence intensities of the resulting hybrids are scanned and translated into gene expression levels. For baker's yeast (*Saccharomyces cerevisiae*) and the dicot *Arabidopsis thaliana*, microarrays containing all genes in the genome are commercially available. Consequently, if RNA is isolated from these organisms after treatment with a fungicide or herbicide, hybridizing the RNA to a microarray can provide information on the response of every gene to that pesticide (*5*). The genes whose expression levels differ significantly from those of the control may indicate the physiological effects of the pesticide, and affected enzymes may provide clues about the metabolic pathway that is targeted.

Different agricultural pesticides with the same mode of action should have similar gene expression patterns, as demonstrated in microarray studies of yeast treated with pharmaceutical fungicides (*6*). Therefore, a database of gene expression profiles in response to known pesticides should help to identify the mode of action of a novel pesticide. An expression profile different from those in the database could indicate a novel molecular target site, whose identity might be partially inferred from the functions of the up- and down-regulated genes. This report describes two microarray studies--one in fungi, and one in plants--of gene expression profiles in response to commercial pesticides or naturally-occurring potential pesticides.

Downloaded by UNIV OF GUELPH LIBRARY on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch020

Gene Expression in *S. cerevisiae* in Response to Sterol Biosynthesis Inhibitors

As a first step to creating a database of gene expression profiles for fungal responses to agricultural fungicides, microarray studies were done with *S. cerevisiae* treated with eight ergosterol biosynthesis inhibitors (sterol biosynthesis inhibitors, or SBI's). Although *S. cerevisiae* is not a plant pathogen, it is a useful model because of its small, fully sequenced genome and the availability of mutants for follow-up studies (*5*), and it has been used in other microarray studies with agricultural fungicides (*7*). SBI's are widely used in both medicine and agriculture because ergosterol is an essential membrane component for fungi (*8*). Those used in agriculture fall into three classes. Class I SBI's inhibit lanosterol C-14 demethylase, which is encoded by *ERG11* and is an early post-squalene intermediate in the ergosterol biosynthetic pathway (Figure 1). Class II SBI's inhibit the C-14 reductase and the Δ8-Δ7-isomerase, which are encoded by *ERG24* and *ERG2*. Class III SBI's inhibit the sterol C-3 keto reductase, which is encoded by *ERG27* and involved in the conversion of 4,4-dimethylzymosterol into zymosterol.

Downloaded by UNIV OF GUELPH LIBRARY on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch020

Materials and Methods

Yeast Strains, Media, and Growth Condition

Cultures of *S. cerevisiae* strain S288C, diluted to an A_{600} of 0.1 from an overnight culture, were grown in synthetic dextrose media (0.17 % yeast nitrogen base without amino acids and ammonium sulfate, 0.5 % ammonium sulfate, 2 % glucose, 165 mM MOPS, pH 7.0) to early log phase (A_{600}=0.2) and then treated with I_{50} concentrations (previously determined) of fungicides dissolved in DMSO or ethanol. Controls received equivalent amounts of solvent (0.5 %, v/v). The Class I SBI's used were prochloraz, triadimefon, imazalil, fenarimol, and fenbuconazole. The Class II SBI's were fenpropimorph and dodemorph. The Class III SBI used was fenhexamid. A putative methionine biosynthesis inhibitor, cyprodinil, was used for comparing SBI gene expression patterns to those induced by a non-SBI fungicide. Cells were harvested after about one doubling time (A_{600} of about 0.4), centrifuged for 5 minutes at 3000 *g* to pellet cells, frozen in liquid nitrogen, and stored at -80 °C until RNA extraction. Duplicates of the harvested cultures were grown for an additional 16-18 hours, to determine if the fungicide concentrations used had indeed caused 50% growth inhibition. Growth was determined as a percentage of the control (set at 100%). Harvested cells were used for microarrays if the growth in the duplicate flasks 18-20 h after treatment was 40-60 % of the control. At least two biological replicates were done for each fungicide, usually on separate days.

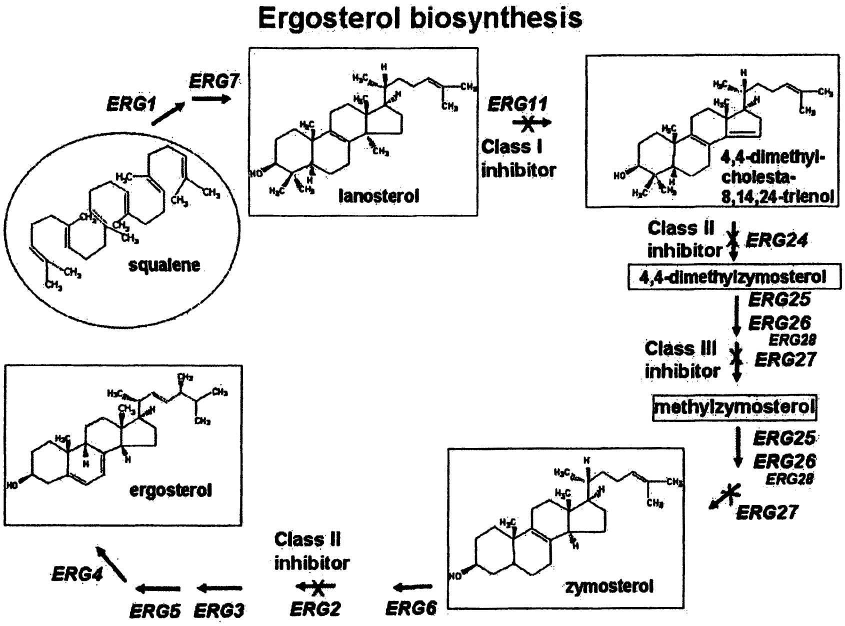

Ergosterol biosynthesis
ERG1
ERG7
squalene
lanosterol
ERG11
Class I inhibitor
4,4-dimethyl-cholesta-8,14,24-trienol
Class II inhibitor
ERG24
4,4-dimethylzymosterol
ERG25
ERG26
Class III inhibitor
ERG28
ERG27
methylzymosterol
ERG25
ERG26
ERG28
ERG27
zymosterol
ERG6
Class II inhibitor
ERG2
ERG3
ERG5
ERG4
ergosterol

Figure 1. Outline of the ergosterol biosynthetic pathway, from squalene to ergosterol. Each arrow represents a separate intermediate, except between 4,4-dimethylzymosterol and zymosterol. Steps inhibited by a Class I, Class II, or Class III SBI are indicated by the "X" on the arrow. Enzymes inhibited: ERG11, lanosterol C-14 demethylase; ERG24, sterol C-14 reductase; ERG27, sterol C-3 keto reductase; ERG2, sterol C-8 isomerase.

RNA Isolation and Microarray Hybridization

Total RNA was isolated with the Qiagen RNeasy Midi-kit, with modifications according to Agarwal et al. (*9*), and mRNA was isolated with the Qiagen Oligotex mRNA kit. Synthesis of cDNA from mRNA, and subsequent labeling of cDNA with Cy3 and Cy5 dyes, were done according to (*10*). Modifications of the original protocol included using an oligo-dT$_{12\text{-}18}$ primer to prime cDNA synthesis. *S. cerevisiae* microarrays from the Ontario Cancer Institute were used for hybridizations. Hybridization and post-hybridization solutions were prepared according to (*11*) (hybridization with formamide; sheared salmon sperm DNA as a blocking agent), except that the final wash

Downloaded by UNIV OF GUELPH LIBRARY on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch020

consisted of 0.1x SSC. The hybridization was done in a GeneTAC hybridization station (Genomic Solutions, Ann Arbor, MI).

Microarray analysis

Arrays were scanned on a ScanArray 5000 confocal laser scanner (Packard Bioscience) with 10 µm resolution using the ScanArray 3.0 software. Each slide was scanned at 550 nm to measure intensity of hybridization to Cy3-coupled cDNA, and at 650 nm to measure intensity of hybridization to Cy5-coupled cDNA. From the two scans, stored as TIFF images, spots were identified and their intensities measured in the QuantArray 3.0 software (Packard Bioscience). Background subtraction and LOWESS sub-grid normalization were done with the GeneTraffic Multi software package from Iobion Informatics (La Jolla, CA; www.iobion.com). The intensity ratio of the fungicide-treated cells to the control was determined for each gene. Annotation of genes was based on the Comprehensive Yeast Genome Database (http://mips.gsf.de/genre/proj/yeast/index.jsp) and the *Saccharomyces* Genome Database (www.yeastgenome.org).

Downloaded by UNIV OF GUELPH LIBRARY on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch020

Results

Since the fungicides within the same SBI class had the same target sites, pooling mean expression levels for the fungicides within a class seemed legitimate. Table 1 shows the mean fold change within each SBI class for six of the ergosterol biosynthetic genes. *ERG11*, the target of the Class I SBI, was significantly upregulated by both Class I and Class II SBI's (Table 1). *ERG2*, whose gene product is one of the targets of the Class II SBI's, was significantly upregulated by Class II SBI's but was not affected by the other fungicide classes tested. Therefore, this response may be a unique characteristic of Class II SBI's. *ERG24*, encoding the other target of the Class II SBI's, was not affected by any of the fungicide classes. Similarly, expression of *ERG 27*, which encodes the target of the Class III SBI, was not affected by any fungicide class.

Although the fungicide classes could not be characterized by up- or downregulation of genes encoding targeted enzymes, the overall patterns of expression provided some differentiation capability. Expression patterns in response to Class I and Class II SBI's were similar for five of the six genes shown in Table 1, while expression patterns in response to the Class III SBI and the methionine biosynthesis inhibitor (MBI) differed markedly from those of the Class I and Class II SBI's. The MBI had no apparent effect on genes of the ergosterol pathway. Surprisingly, the class III inhibitor also had very little effect, suggesting that the toxicity of fenhexamid to *S. cerevisiae* may be due to some mechanism other than inhibition of ergosterol biosynthesis. These results suggest that looking at expression of genes in candidate biosynthetic pathways can help identify the molecular target of a fungicide.

Table 1. Fold change in expression (relative to the control) of some ergosterol biosynthesis genes of *S. cerevisiae* exposed to Class I, II, or III SBI's. Means plus or minus standard errors are shown for SBI classes I and II. For SBI Class III and the MBI, because only one fungicide was examined within each class, means plus or minus standard deviations for the within-fungicide replicates are shown.

	- Gene -					
SBI class	***ERG11***	***ERG24***	***ERG27***	***ERG6***	***ERG2***	***ERG3***
Class I (N=5)	**2.14 ± 0.40**	**0.90 ± 0.05**	**1.61 ± 0.09**	**2.26 ± 0.44**	**1.51 ± 0.16**	2.99 ± 0.43
Class II (N=2)	**3.07 ± 0.37**	**0.94 ± 0.06**	**1.95 ± 0.08**	**3.36 ± 0.14**	**2.04 ± 0.56**	3.57 ± 0.59
Class III (N=1)	**1.18 ± 0.21**	**0.90 ± 0.09**	**1.30 ± 0.11**	**1.11 ± 0.20**	**1.11 ± 0.40**	0.87 ± 0.13
MBI (N=1)	0.85 ± 0.01	0.90 ± 0.14	1.25 ± 0.12	1.10 ± 0.19	0.828 ± 0.238	1.25 ± 0.094

Gene Expression in Arabidopsis in Response to Benzoxazolin-2(3H)-one (BOA)

Benzoxazolin-2(3H)-one, or BOA (Figure 2), is an allelochemical and anti-pathogen/anti-insect compound produced by some grasses such as wheat, maize, and rye, as well as by some plants in other families (*12*). Arabidopsis was used as a model to better understand the responses of plants exposed to BOA.

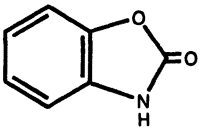


Figure 2. Benzoxazolin-2(3H)-one (BOA).

Downloaded by UNIV OF GUELPH LIBRARY on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch020

Materials and Methods

Plant Material, Growth Conditions, and Treatments

Determination of inhibitory concentrations, and treatment for microarray/PCR studies, were done on A. thaliana (Columbia) seedlings grown from surface-sterilized seeds that were cold-treated for three days before transfer for 10 days to a growth chamber at 21oC under a 16-hour photoperiod and light intensity of 150 µmol m-2 s-1.

The I50 and I80 concentrations of BOA were determined by measuring primary root lengths of seedlings grown on 1.0 % MS agar (0.5x MS salts, 1x Gamborg's B-5 vitamins, 1.0% sucrose, pH 5.7), in dishes kept in a vertical position during growth. For microarray studies, seedlings were germinated inside of tissue culture vessels with vented lids, on 0.3-µm membrane rafts supported by buoyant floats on liquid MS media. BOA, other xenobiotics, or solvent-only control solutions were added to the medium after 10 days, and the seedlings were returned to the growth chamber and harvested 24 hours post-treatment. Seedlings, removed from the raft as a single mat, were frozen in liquid nitrogen and stored at -80 °C.

Downloaded by UNIV OF GUELPH LIBRARY on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch020

RNA isolation

For microarray analysis, total RNA was isolated from 0.5 g of frozen pulverized tissue, using the Trizol reagent (Invitrogen Corporation, Carlsbad, CA), and further purifying the isolated RNA with the Qiagen RNeasy Plant Mini-Kit. Total RNA for real-time PCR analysis was isolated with the RNeasy Plant Mini-Kit, using 50 mg of frozen pulverized tissue.

Microarray hybridization, Real-Time PCR, and Analysis

RNA was hybridized to the Affymetrix Arabidopsis ATH1 Genome Array, using the Affymetrix protocols. For real-time PCR, first-strand cDNA synthesized from 2 µg total RNA was amplified on a GenAmp® 5700 Sequence Detection System (Applied Biosystems). All reactions were performed in triplicate, using the SYBR Green Master Mix (Applied Biosystems). The same cDNA was used, in separate reactions, with primers specific to a gene of interest and primers for the 18S ribosomal RNA. PCR conditions consisted of 10 min of denaturation (95 °C), followed by 40 cycles of a 15-sec denaturation at 95 °C and a 1-min annealing/elongation at 60 °C. Fold change in gene expression relative to the control treatments was determined with the $\Delta\Delta C_T$ method (*13*).

Results

The BOA concentrations causing 50% and 80% inhibition of root growth on 10-day-old Arabidopsis seedlings were 540 μM and 1 mM, respectively. These concentrations were used when treating hydroponically-grown seedlings for microarray analysis.

In the I_{50} or I_{80} treatments, some genes responded to BOA treatment with at least a 2.5-fold increase in expression, relative to the control. These were classified under 13 functional categories designated by the MIPS *Arabidopsis thaliana* database (*14*): protein degradation; development; lipid, fatty acid, and isoprenoid metabolism; cellular biogenesis; energy generation; amino acid metabolism; carbohydrate metabolism; transcription; small molecule transport; signal transduction; other metabolism; detoxification, cell rescue, and defense; and unknown. The largest category into which responsive genes fell was that of "unknown function," followed by "detoxification, cell rescue, and defense," and "other metabolism" (which included genes from the detoxification category because detoxification can involve biosynthesis of new molecules or degradation of existing ones). Thus, most of the identifiable genes had functions associated with response to a xenobiotic.

As shown in Table 2, genes from the three recognized phases of detoxification (*15*) responded to BOA treatment. Genes possibly involved in transforming xenobiotics by oxidation or reduction (Phase I) included aldo-keto reductases, peroxidases, and cytochrome P450 monooxygenases. Genes that could be involved in conjugating the transformed xenobiotics to other molecules (Phase II), making them more water-soluble and capable of sequestration or excretion, included glutathione transferases and glucosyltransferases. ABC transporters, possibly involved in compartmentation of conjugated xenobiotics (Phase III) were also induced.

Table 2. Some classes of genes induced at least 2.5-fold by I_{50} or I_{80} concentrations of BOA.

Detoxification Phase	Gene descriptor	Number of genes induced in this class
Phase I (transformation)	Also-keto reductase Cytochrome P450 Peroxidase	4 6 6
Phase II (conjugation)	Glutathione transferase Glucosyltransferase	7 11
Phase III (compartmentalization)	ABC transporter	6

Downloaded by UNIV OF GUELPH LIBRARY on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch020

To validate the microarray data, results for 16 of the responsive genes were confirmed by quantitative real-time PCR, using gene-specific primers and quantifying gene expression by measuring the fluorescence intensity of SYBR Green dye, which fluoresces in the presence of double-stranded DNA, during each cycle of cDNA amplification (*13*). Responsiveness to BOA was confirmed for all 16 genes (not shown), and fold changes in the real-time PCR assays were quite similar to fold changes in the microarray experiments.

Summary and Conclusions

Microarrays, by capturing a profile of gene expression under given conditions, can provide information on the effects of candidate pesticides. They may help to identify possible modes of action, or they may provide new information about processes occurring in the target organism upon exposure. Although the results should not be relied upon without further validation such as real-time PCR, work with known mutants, or biochemical studies, they can serve as a guidepost for finding new information. As such, microarrays should be a useful tool in future studies of natural products as candidate pesticides.

Downloaded by UNIV OF GUELPH LIBRARY on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch020

References

1. Leroux, P.; Fritz, R.; Debieu, D.; Albertini, C.; Lanen, C.; Bach, J.; Gredt, M.; Chapeland, F. *Pest Manag. Sci.* **2002**, *58*, 876-888.
2. Mercer, E. I. *Lipids* **1991**, *26*, 584-597.
3. Lockhart, D.J.; Winzeler, E.A. *Nature* **2000**, *405*, 827-836.
4. Wodicka, L.; Dong, H.; Mittmann, M.; Ho, M.-H.; Lockhart, D. J. *Nature Biotech.* **1997**, *15*, 1359-1367.
5. Jia, M. H.; Larossa, R. A.; Lee, J.-M.; Rafalski, A.; DeRose, E.; Gonye, G.; Xue, Z. *Physiol. Genomics* **2000**, *3*, 83-92.
6. Bammert, G. F.; Fostel, J. M. *Antimicrob. Agents Chemother.* **2000**, *44*, 1255-1265.
7. Kitagawa, E.; Momose, Y.; Iwahashi, H. *Environ. Sci. Technol.* **2003**, *37*, 2788-2793.
8. Daum, G.; Lees, N. D.; Bard, M.; Dickson, R. *Yeast* **1998**, *14*, 1471-1510.
9. Agarwal, A. K.; Baerson, S. R.; Rogers, P. D.; Jacob, M. R.; Barker, K. S.; Cleary, J. D.; Walker, L. A.; Nagle, D. G.; Clark, A. M. *J. Biol. Chem.* **2003**, *278*, 34998-35015.
10. Protocol from The Institute for Genomic Research (TIGR) (http://www.tigr.org/tdb/microarray/protocolsTIGR.shtml).

11. Corning GAPS II Coated Slides Instruction Manual (available in pdf format at www.corning.com/lifesciences), p. 7.
12. Schulz, M.; Wieland, I. *Chemoecol.* **1999**, *9*, 133-141.
13. Applied Biosystems GeneAmp 5700 User's Manual.
14. MIPS *Arabidopsis thaliana* database; http://mips.gsf.de/proj/thal/db/
15. Sandermann, H. Jr. In *Plant Biotechnology and In Vitro Biology in the 21st Century*; Altman, A., Ed.; Kluwer Academic Publishers: Netherlands, 1999; pp 321-328.

Downloaded by UNIV OF GUELPH LIBRARY on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch020

Downloaded by YORK UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch021

Chapter 21

A Functional Genomics Approach for the Identification of Genes Involved in the Biosynthesis of the Allelochemical Sorgoleone

Scott R. Baerson[1], Franck E. Dayan[1], Agnes M. Rimando[1], Zhiqiang Pan[1], Daniel Cook[1], N. P. Dhammika Nanayakkara[2], and Stephen O. Duke[1]

[1]Natural Products Utilization Research Unit, Agricultural Research Service, U.S. Department of Agriculture, P.O. Box 8048, University, MS 38677
[2]National Center for Natural Products Research, School of Pharmacy, University of Mississippi, University, MS 38677

Several *Sorghum* species are allelopathic, producing phytotoxins such as sorgoleone and its analogues. Sorgoleone accounts for much of this phytotoxicity, representing up to 90% (w/w) of the content of *Sorghum bicolor* root exudates. Previous and ongoing studies suggest that the biosynthetic pathway for this compound involves a polyketide synthase activity utilizing atypical long chain fatty acyl-CoA starter units, resulting in the addition of a quinone head via iterative extender unit condensations. An expressed sequence tag (EST) study was performed using random sequences from a *Sorghum bicolor* root hair-specific cDNA library, and highly expressed candidate sequences representing all of the putative enzyme classes required for the sorgoleone biosynthetic pathway were identified.

In Natural Products for Pest Management; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

Natural products, well known as a source of novel pharmaceuticals, are also a promising source of novel herbicides *(1,2)*. Many plant species have been shown to produce such phytotoxic secondary metabolites, some of which may play a direct role in allelopathic interactions. Natural products such as allelochemicals offer an attractive alternative to synthetic herbicides, as they are generally thought to be more environmentally and toxicologically friendly. In addition, several studies indicate that many allelochemicals and other natural phytotoxins may inhibit molecular target sites distinct from those targeted by commercially available herbicides *(2)*.

Sorgoleone (2-hydroxy-5-methoxy-3-[(*Z*,*Z*)-8',11',14'-pentadecatrienyl]-p-benzoquinone (Figure 1), is an allelochemical produced by several *Sorghum* species which offers promise as a natural pesticide. Sorgoleone refers to a group of structurally related benzoquinones having a hydroxy and a methoxy substitution at positions 2 and 5, respectively; and either a 15- or 17-carbon chain with 1, 2, or 3 double bonds at position 3 *(3)*. These quinones were first isolated from hydrophobic root exudates of *Sorghum bicolor* and are distinguished from one another on the basis of their molecular weights. The 2-hydroxy-5-methoxy-3-[(*Z*,*Z*)-8',11',14'-pentadecatrienyl]-*p*-benzoquinone (Figure 1) accounts for more than 80% of the root exudates of *Sorghum*, and is released into the soil where it represses the growth of other plants present in their surroundings. The remaining exudates components consist of sorgoleone congeners differing in the length or degree of unsaturation of the 3-alkyl side chain, and in the substitution of the quinone head *(4,5,6)*. As the sorgoleone-containing droplets are found exclusively on the root hairs *(7)*, it is likely that most, if not all, of the biosynthetic pathway of sorgoleone is compartmentalized in root hairs. Ultrastructural studies indicate that these cells are highly physiologically active, containing a great number of mitochondria and the presence of a dense endomembrane system *(7)*. Our current efforts have been focused on the isolation of genes encoding biosynthetic enzymes for this pathway using expressed sequence tags (ESTs).

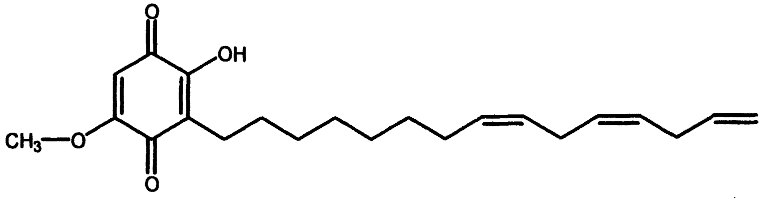


Figure 1. Chemical structure of sorgoleone (2-hydroxy-5-methoxy-3-[(Z,Z)-8',11',14'-pentadecatrienyl]-p-benzoquinone).

Downloaded by YORK UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch021

Materials and Methods

cDNA Library Construction and EST Data Analysis

Sorghum bicolor (cv. BTX623) were grown for 5-7 days on a capillary mat system as described previously *(7)*. Root hairs were then isolated by the method of Bucher et al. *(8)*, and total RNAs were isolated using the Trizol reagent (Invitrogen Corporation, Carlsbad, CA) per manufacturer's instructions. Tissue disruption was performed using a hand-held homogenizer at 25,000 rpm. RNA purity was determined spectrophotometrically, and integrity was assessed by agarose gel electrophoresis. Poly-A+ mRNAs were prepared using an Oligotex mRNA Midi Kit (Qiagen, Valencia, CA), then used for the construction of the cDNA library with a Uni-Zap XR cDNA library construction kit (Stratagene, La Jolla, CA). Mass excision of the primary library was performed to generate phagemid clones, which were then randomly selected for sequencing via the University of Georgia, Laboratory for Genomics and Bioinformatics wet-lab pipeline *(9)*. Raw sequence traces were then filtered for quality control and elimination of contaminating vector sequences via an automated bioinformatics pipeline developed at the University of Georgia *(9)*. Database mining was performed using the Magic Gene Discovery software (L. Pratt, University of Georgia, unpublished results), and by BLASTN and TBLASTN analysis *(10)*.

Downloaded by YORK UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch021

Real-Time RT-PCR

Total RNAs were prepared using Trizol as described above, then re-purified using an RNeasy Midi Kit (Qiagen, Valencia, CA), including an "on-column" DNase I treatment to remove residual DNA contamination. Real-time PCR was performed in two biological replicates (i.e., two RNA samples from different plants, with three PCR reactions on each RNA sample) for each tissue using an ABI PRISM™ 5700 Sequence Detector (Applied Biosystems, Foster City, CA) with gene-specific primers and primers specific to 18S rRNA (Forward, 5'- GGCTCGAAGACGATCAGATACC-3'; reverse, 5'-TCGGCATCGTTTATGGTT- 3'). First-strand cDNAs were synthesized from 2 µg of total RNA in a 100 µl reaction volume using a TaqMan Reverse Transcription Reagents Kit (Applied Biosystems, Foster City, CA) and random hexamers as primer. For PCR reactions using gene-specific primers, the cDNA was diluted 50-fold and 2.5 µl (~0.5 ng cDNA) was used for a 25 µl PCR reaction. For PCR reactions using 18S rRNA-specific primers, the cDNA was diluted 50,000-fold and 2.5 µl (~0.5 pg cDNA) was used for a 25 µl PCR reaction. The real-time PCR reactions were performed using the SYBR Green PCR Master Mix Kit (Applied Biosystems, Foster City, CA) with denaturation at

95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec, and annealing/extension at 60°C for 1 min. For the 18S rRNA assays, the primers were at 50 nM each and for the gene-specific assays, the primers were at 450 nM each. The changes in fluorescence of SYBR Green I dye in every cycle were monitored by the ABI 5700 system software and the threshold cycle (CT) for each reaction was calculated. The relative amount of PCR product generated from each primer set was determined based on the C_T value. 18S ribosomal RNA was used as the normalization control for all assays. The C_T value of 18S rRNA was subtracted from that of the gene-specific value to obtain a ΔC_T value, and then the ΔC_T value for the lowest-expressing tissue from that of each tissue to obtain a $\Delta\Delta C_T$ value. The gene expression level in a tissue relative to that in the lowest-expressing tissue was expressed as $2^{-\Delta\Delta C_T}$.

Downloaded by YORK UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch021

Heterologous Expression of Recombinant *O*-methyltransferases

Full-length coding sequences were determined in some cases using full-length cDNA clones from the root hair cDNA library (described above), and by assembly with 5' clones identified in the public sorghum EST data. When necessary, 5' ends were also generated using the SMART RACE cDNA Amplification Kit (Clontech Laboratories Inc., Palo Alto, CA) per manufacturer's instructions. PCR products containing complete open reading frames flanked by appropriate restriction sites, were then generated and directly cloned into pET15b (EMD Biosciences, La Jolla, CA), in-frame with a poly-histidine tract and thrombin cleavage site. The resulting *E. coli* expression vectors were transformed into strain BL21/DE3 (EMD Biosciences, La Jolla, CA) for recombinant enzyme studies.

For recombinant *O*-methyltransferase production, *E. coli* cultures were grown at 37°C to an optical density of 0.6 at 600 nm, then induced with 0.5 mM IPTG and allowed to grow 5 additional hours at 25°C. Cells were harvested by centrifugation at approximately 3000 x g for 20 min at 4°C, washed with cold 0.9% NaCl, then collected by re-centrifugation at 3000 x g. Pellets were resuspended in cold lysis buffer (50 mM Tris-HCl, pH 7.5, 1 M NaCl, 5 mM imidazole, 10% glycerol, 1 μg/ml leupeptin), and extracted using a French Press at a pressure of 1500 p.s.i. Benzonase (25 U/ml) and 1 mM PMSF were added immediately to the lysate. After 15 min incubation at room temperature, the lysate was centrifuged at 15,000 x g for 20 min, and supernatant was loaded onto a Ni-column activated with 2 ml of 0.1 M $NiSO_4$ and washed with 10 ml of distilled water. The Ni-column was previously equilibrated with 10 ml buffer A (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 5 mM imidazole). The column was washed with 4 ml buffer A between each 2 ml of supernatant. Once all of the sample was loaded, the column was washed with 8 ml of buffer A followed with

8 ml of buffer B (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 100 mM imidazole). Recombinant *O*-methyl transferases were then eluted with 2.5ml of elution buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 250 mM imidazole). The column was washed with 10 ml wash buffer C (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 M imidazole), followed by 10 ml of distilled water to remove any remaining proteins. The recombinant protein-containing fraction (500 mM imidazole) was desalted on a PD-10 column equilibrated with cold desalting buffer (20 mM Tris-HCl, pH 7.5, 10mM DTT, 10% glycerol). Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Enzyme preparations were stored at –80°C prior to use.

O-methyltransferase Enzyme Assays

O-methyltransferase assays were performed essentially as described by Wang and Pichersky *(11)*, except that a mixture of ethyl acetate:hexane (1:1) was substituted for 100% ethyl acetate for extraction of enzyme reactions prior to scintillation counts.

Downloaded by YORK UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch021

Results and Discussion

Our current understanding of the sorgoleone biosynthetic pathway *(12)* indicates that three different classes of enzyme, i.e. a minimum of three genes, would be required for biosynthesis starting with an acyl-CoA starter molecule and malonyl-CoA (Figure 2). In addition, the proposed C16:0-CoA precursor would require a novel fatty acid desaturase to generate the Δ-9,12,15 double bond configuration shown in Figure 2. We have therefore targeted fatty acid desaturases, polyketide synthases, *O*-methyltransferases, and cytochrome P450s from *Sorghum bicolor* for obtaining candidate sequences for subsequent biochemical studies, in our search for sorgoleone biosynthetic enzymes.

Given the likelihood that sorgoleone is synthesized predominantly in root hair cells *(7)*, it follows that the biosynthetic enzymes are exclusively or predominantly localized in this cell type. Taking the significant quantity of sorgoleone-containing exudate produced by these cells into account, it is also reasonable to speculate that the corresponding mRNAs encoding these enzymes would be among the most abundant. Expressed sequence tag (EST) analysis was selected for our gene isolation strategy, as this approach is ideally suited for profiling the more abundant transcripts in a specific cell or tissue type *(13)*.

Downloaded by YORK UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch021

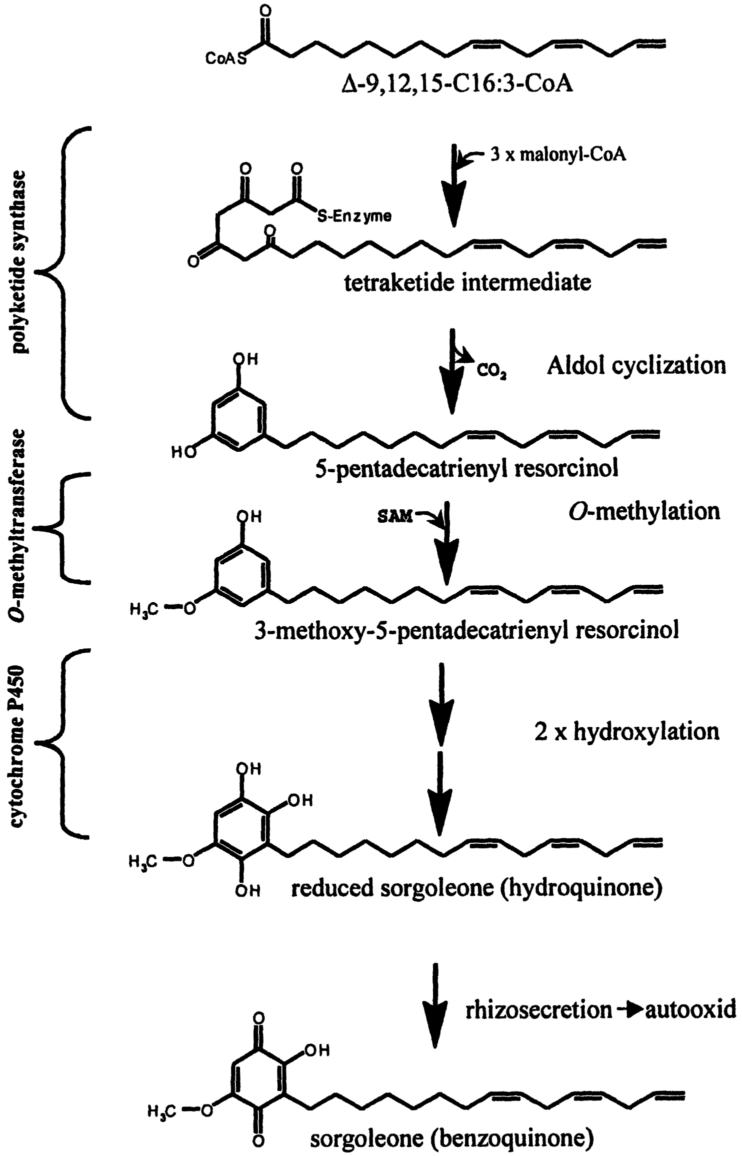

CoAS
Δ-9,12,15-C16:3-CoA
polyketide synthase
3 x malonyl-CoA
S-Enzyme
tetraketide intermediate
CO₂
Aldol cyclization
5-pentadecatrienyl resorcinol
O-methyltransferase
SAM
O-methylation
3-methoxy-5-pentadecatrienyl resorcinol
cytochrome P450
2 x hydroxylation
reduced sorgoleone (hydroquinone)
rhizosecretion → autooxid
sorgoleone (benzoquinone)

Figure 2. Proposed biosynthetic pathway for sorgoleone. The hydroquinone, produced in vivo, undergoes autooxidation once secreted into the rhizophere to yield the benzoquinone, sorgoleone.

The *S. bicolor* cultivar (BTX623) was chosen for our experimental system, since the majority of the over 180,000 public *S. bicolor* ESTs were derived from this cultivar *(14)*. To isolate large quantities of root hair tissue, a capillary mat system was employed as described by Czarnota and co-workers *(7)*, to promote vigorous soil-free root system development and exudate production with *S. bicolor* seedlings. Root tissues were harvested and stirred gently in liquid nitrogen, then filtered through a 250 µM mesh as previously described *(8)*. The procedure, which was originally used for isolation of tomato root hair cells, also yielded highly-enriched root hair cell preparations from BTX623 (Figure 3).

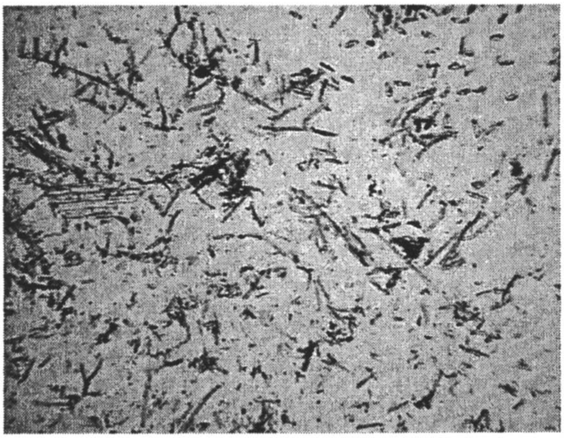


Figure 3. Root hair cell isolation. Bright-field light micrograph of S. bicolor root hair cells prepared by treating root tissue with liquid nitrogen, followed by 250 µM filtration as described by Bucher et al. (8).

RNA extracted from this material was used for the construction of a directional cDNA library, from which an EST database was generated. For the EST database, 6,624 plasmid minipreps from sixty-nine 96-well plates of bacteria were prepared, and 5' sequencing reactions were performed on all 6,624. Of these attempts, 82.56% were successful, yielding 5,469 quality 5' ESTs. The average EST length, using a moving window with a Phred quality score of 16, is 451 bp (a Phred quality score of 16 corresponds to approximately 97.5% accuracy). This average was calculated after excluding vector and adapter, as well as low quality sequence ends.

To mine the EST data for candidate fatty acid desaturase, polyketide synthase, *O*-methyltransferase, and cytochrome P450-like sequences, we used both the Magic Gene Discovery software *(9)*, and also performed BLAST

Downloaded by YORK UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch021

searches using functionally-characterized protein sequences as queries against the EST dataset translated in all possible reading frames. From these analyses, 47 fatty acid desaturase, 9 polyketide synthase, 94 methyltransferase, and 21 P450-like ESTs were identified (Table I). Surprisingly, the methyltransferase-like sequences comprised the largest group, representing approximately 1.7% of all sequences in the data set. Typically in plants, cytochrome P450-like sequences are the most highly represented within the genome among these four enzyme classes. For example, in *Arabidopsis thaliana*, which has a relatively compact genome among plant species, as many as 286 genes comprise a superfamily of P450-related sequences *(15)*. The observed distribution in sorghum root hairs most likely reflects the highly specialized metabolism occurring within this cell type.

Downloaded by YORK UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch021

Table I. Summary of candidate sequences identified in *S. bicolor* root hair EST database

Family	*Clones*	*Contigs*	*Singletons*	*%,Total*	*Root hair-specific*
Desaturase	47	11	4	0.859	3
Polyketide synthase	9	5	3	0.165	3
Methyltransferase	94	23	12	1.72	≥3[a]
P450	21	18	15	0.384	4

[a] Three sequences exhibiting root hair-specific expression were identified from a subset of the 23 methyltransferase-like contigs, therefore the total number of root hair-specific methyltransferases represented in the data may be greater than three.

A secondary screen using real-time PCR was also employed to eliminate sequences whose expression pattern did not correlate with the accumulation of sorgoleone. Gene-specific primers were designed for all of the contigs identified (Table I), with the exception of methyltransferases, where analysis was performed only on a subset of the 23 contigs. For all of the targeted enzyme families, 3-4 candidate genes were identified which exhibited the highest expression levels in root hairs (Table I). For all root hair-specific candidates, full length coding sequences were generated by PCR and subcloned into *E. coli* expression vectors, or for cytochrome P450-like sequences, vectors engineered for heterologous expression in *Saccharomyces cerevisiae*.

As shown in Table I, three O-methyltransferase candidate sequences were identified exhibiting root hair-preferential expression patterns. Full-length open reading frames for all three were overexpressed in *E. coli*, and purified using an activated Ni-column (see Materials and Methods). Various benzene-derivatives containing different functional groups and substitution patterns were tested in enzyme assays with all three recombinant enzymes *(16)*, including a series of 5-substituted alkyl-resorcinols (Figure 4).

Downloaded by YORK UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch021

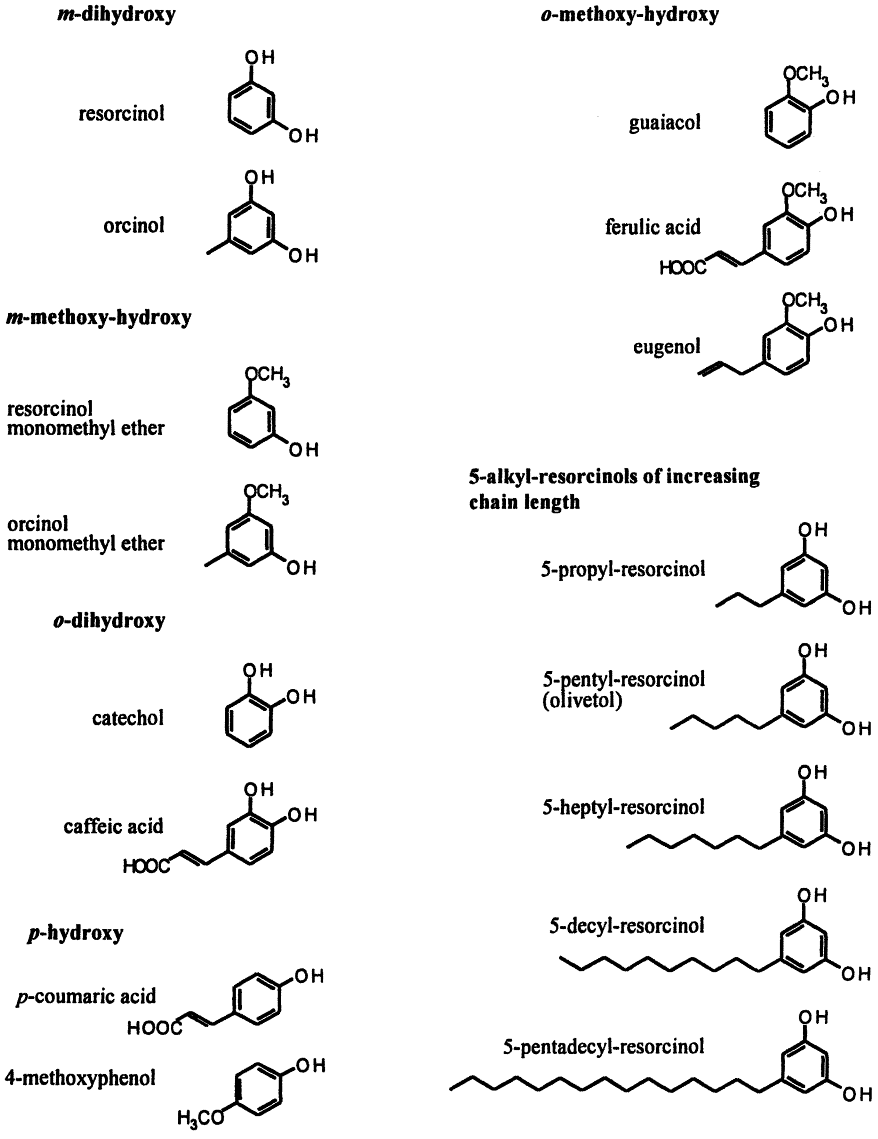

m-dihydroxy
resorcinol
OH
OH
orcinol
OH
OH
m-methoxy-hydroxy
resorcinol monomethyl ether
OCH3
OH
orcinol monomethyl ether
OCH3
OH
o-dihydroxy
catechol
OH
OH
caffeic acid
OH
OH
HOOC
p-hydroxy
p-coumaric acid
OH
HOOC
4-methoxyphenol
OH
H3CO
o-methoxy-hydroxy
guaiacol
OCH3
OH
ferulic acid
OCH3
OH
HOOC
eugenol
OCH3
OH
5-alkyl-resorcinols of increasing chain length
5-propyl-resorcinol
OH
OH
5-pentyl-resorcinol (olivetol)
OH
OH
5-heptyl-resorcinol
OH
OH
5-decyl-resorcinol
OH
OH
5-pentadecyl-resorcinol
OH
OH

Figure 4. O-methyltransferase substrate specificity. Structures are shown for some of the substrates used for determining specificity of recombinant S. bicolor *O-methyltransferases.*

Significantly, one of the three *O*-methyltransferase clones (clone G10_84), preferentially utililized 5-substituted alkyl-resorcinols among all of the substrates analyzed. 5-Pentadecatrienyl resorcinol (Figure 2), the proposed *in vivo* substrate for the *O*-methyltransferase involved in sorgoleone biosynthesis, is closely related to these compounds, thus G10_84 could represent a gene which participates in this pathway. The predicted protein sequence of G10_84 exhibits a high degree of similarity to ZRP4 of corn (Table II), a putative *O*-methyltransferase preferentially expressed in root endodermal cells *(17)*. Of significance, among all previously characterized plant enzymes, G10_84 is most closely related to an orcinol-specific (5-methyl-resorcinol-specific) *O*-methyltransferase identified from *Rosa hybrida (16)*. Gene knock-out studies

Downloaded by YORK UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch021

Table II. Top 10 BLASTP results for clone G10_84 using the NCBI non-redundant (nr) peptide sequence database

Accession	*Description*	[a]*Score*	[b]*E-value*
AAU03114.1	putative o-methyltransferase ZRP4 [*Oryza sativa* (japonica cultivar-group)]	378	1e-103
AAU03113.1	putative o-methyltransferase ZRP4 [*Oryza sativa* (japonica cultivar-group)]	376	1e-103
P47917/ZRP4 _MAIZE	O-methyltransferase ZRP4 – maize	360	3e-98
BAD29092.1	flavonoid 7-O-methyltransferase-like [*Oryza sativa* (japonica cultivar-group)]	335	1e-90
NP_919602.1	putative o-methyltransferase ZRP4 [*Oryza sativa* (japonica cultivar-group)]	324	2e-87
NP_919605.1	putative o-methyltransferase ZRP4 [*Oryza sativa* (japonica cultivar-group)]	319	9e-86
BAD37839.1	putative o-methyltransferase ZRP4 [*Oryza sativa* (japonica cultivar-group)]	310	4e-83
XP_481333.1	putative catechol o-methyltransferase ZRP4 [*Oryza sativa* (japonica cultivar-group)]	309	7e-83
AAM23004.1	orcinol O-methyltransferase [*Rosa hybrid* cultivar]	305	8e-82
XP_477999.1	putative o-methyltransferase [*Oryza sativa* (japonica cultivar-group)]	305	1e-81

[a] The score (S) for an alignment is calculated by summing the scores for each aligned position and the scores for gaps.

[b] The E-value indicates the number of different alignments with scores equivalent to or better than (S) that are expected to occur in a database search by chance. The lower the E-value, the more significant the score.

with transgenic sorghum plants will be pursued in future efforts, which could provide further evidence that this gene encodes a key enzyme in the sorgoleone biosynthetic pathway.

Summary and Conclusions

Our strategy to identify genes involved in sorgoleone biosynthesis hinges on the analysis of expressed sequence tags. Toward this end, an annotated database consisting of 5,469 5' EST sequences has been generated, using the same cultivar (BTX623) that the majority of the current sorghum genomics infrastructure has been based on. Currently, we plan to make the data publicly available in GenBank by April, 2005. Thus, in addition to providing a source of candidate sequences for the present study, the root hair EST data will directly augment the existing public sorghum data, and furthermore, expand our understanding of the physiology and genetics of a highly specialized and unique cell type.

As a gene isolation strategy, pairing EST analysis with high-throughput expression analysis has been highly efficient at identifying candidate sequences preferentially expressed in *S. bicolor* root hair cells (Table I). This has led to significant progress made thus far in the identification of an *O*-methyltransferase gene potentially involved in sorgoleone biosynthesis. These, and similar efforts, may ultimately provide the tools for the future development of novel allelopathic crop species that are less reliant on synthetic herbicides *(18)*.

Downloaded by YORK UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch021

Literature Cited

1. Dayan, F. E. In *Encyclopedia of Pest Management*; Pimentel, D. Ed; Marcel Dekker, Inc.: New York, NY, 2002; pp 521-525.
2. Duke, S.O.; Rimando, A.M.; Baerson, S.R.; Scheffler, B.E.; Ota E.; Belz, R.G. *J. Pesticide Sci.* **2002,** *27,* 298-306.
3. Netzly, D.; Riopel, J.L.; Ejeta, G.; Butler, L.G. *Weed Sci.* **1988,** *36,* 441-446.
4. Kagan, I.A.; Rimando, A.M.; Dayan, F.E. *J. Agric. Food Chem.* **2003,** *51,* 7589-7595.
5. Rimando, A.M; Dayan, F.E.; Czarnota, M.A.; Weston, L.A.; Duke, S.O. *J. Nat. Prod.* **1998,** *61,* 927-930.
6. Rimando, A.M.; Dayan, F.E.; Streibig, J.C. *J. Nat. Prod.* **2003,** *66,* 42-45.

7. Czarnota, M.A.; Paul, R.N.; Weston, L.A.; Duke, S.O. *Internat. J. Plant Sci.* **2003**, *164*, 861-866.
8. Bucher, M.; Schroeer, B.; Willmitzer, L.; Riesmeier, J.W. *Plant Mol. Biol.* **1997**, *35*, 497-508.
9. http://fungen.botany.uga.edu
10. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. *J. Mol. Biol.* **1990**, *215*, 403-410.
11. Wang, J.; Pichersky, E. *Arch. Biochem. Biophys.* **1999**, *368*, 172-180.
12. Dayan, F.E.; Kagan, I.A.; Rimando, A.M. *J. Biol. Chem.* **2003**, *278*, 28607-28611.
13. Boguski, M.S.; Lowe, T.M.; Tolstoshev, C.M. *Nat. Genet.* **1993**, *4*, 332-333.
14. http://www.tigr.org
15. Xu, W.; Bak, S.; Decker, A.; Paquette, S.M.; Feyereisen, R.; Galbraith, D.W. *Gene.* **2001**, *272*, 61-74.
16. Lavid, N.; Wang, J.; Shalit, M.; Guterman, I.; Bar, E.; Beuerle, T.; Menda, N.; Shafir, S.; Zamir, D.; Adam, Z.; Vainstein, A.; Weiss, D.; Pichersky, E.; Lewinsohn, E. *Plant Physiol.* **2002**, *129*, 1899-1907.
17. Held, B.M.; Wang, H.; John, I.; Wurtele, E.S.; Colbert, J.T. *Plant Physiol.* **1993**, *102*, 1001-1008.
18. Duke, S.O. *Trends Biotechnol.* **2003**, *21*, 192-195.

Downloaded by YORK UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch021

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch022

Chapter 22

Regulatory Considerations for Researchers of Natural Products in the United States

M. P. Braverman, D. L. Kunkel, J. J. Baron and R. H. Holm

**IR-4 Project, Rutgers, The State University of New Jersey,
681 U.S. Highway 1 South, North Brunswick, NJ 08902–3390**

This chapter serves as an aid for scientists involved in university and governmental research programs on how to integrate their observations and discussions with other professionals for satisfying regulatory requirements as a supplement to their research projects. The regulatory considerations in transitioning from a basic research project into a marketable product are described, including (1) the registration process, (2) data requirements, and (3) providing rationale and support from acceptable literature sources in place of generating new studies. Information on institutional infrastructure related to product development is also provided.

Introduction

Numerous organisms and biochemicals with pest control activities are often discovered and investigated by university and governmental scientists who follow a process of proposal, research, and publication, without considering if their endeavors have any potential for further utilization. While some scientists are concerned with transcending beyond this process, most discoveries end with

In Natural Products for Pest Management; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

a journal publication. Scientists who do consider developing their discoveries for practical use often delay publication. By not considering development sooner, many opportunities to assist in the regulatory process, known as registration, are missed. Even those scientists who may not be immediately interested in pursuing commercialization should consider if their products have significant regulatory obstacles. Harpin, a protein from a plant pathogen, *Erwinia amylovara,* that produces fire blight in apples and pears, induces a plant to mobilize its own defenses against pathogens and insects. The discovery of the Harpin protein was reported (*1*) more than 8 years prior to its registration. Once commercial interests began, Harpin was registered as a biopesticide in 2000 (*2*).

Bench scientists could benefit from interacting with applied scientists and others in related fields in determining the need for a product, evaluating its efficacy in relation to existing products, and estimating market potential. Many observations appear promising in petri dish or greenhouse experiments but may fail under field conditions or unanticipated high costs may also negate further practical development.

In order to succeed, new products must either fill a critical void in crop protection strategy or have a fit with conventional agriculture if it is going to be commercially viable. In their reviews of commercial development, Hofstein and Chapple (*3*) and Bowers (*4*) discussed commercial development and provide insights into assessing product potential. At some point in the process, a professional registration consultant or IR-4 may need to be involved in order to prepare the information for review by the U.S. Environmental Protection Agency (EPA).

While making observations or while interpreting data, a researcher observes that the studies reveal some unusual effects on a biological entity. Possibly inhibition of growth or a repelling (attracting) of another species. Is this biological activity reproducible? Is it measurable? Is this possibly a biopesticide? If so, is it worth pursuing? This chapter focuses on the regulatory aspects of natural products as biopesticides and how public sector researchers can assist with satisfying some of the regulatory requirements.

Natural Products as Biopesticides

While the term "natural product" is readily understood by today's chemists, the term has many meanings when several governmental agencies have statutory obligations with overlapping responsibilities (see Table 1). Nakanishi (*5*) has

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch022

stated "Except for minerals, our surroundings consist of entirely organic natural products, either of pre-biotic origins or from microbial, plant, or animal sources." For this discussion, however, "natural product" has been restricted to chemical products classified by the EPA as biopesticides.

Table 1. Department and Agencies with Oversight Affecting Pesticides

Health and Human Services	CDC FDA	Center for Disease Control and Prevention Food and Drug Administration
U.S. Department of Agriculture	ARS APHIS CSREES IR-4 NOP	Agricultural Research Service Agriculture Plant Health and Inspection Service Cooperative State Research, Education and Extension Services IR-4 Project National Organic Program
U.S. Department of Commerce	NOAA	National Oceanic and Atmospheric Administration Estuaries, Coastal Waters and Oceans National Marine Fisheries Service
U.S. Department of the Interior	F&WS USGS	Fish and Wildlife Service Endangered Species Invasive Species U.S. Geological Survey Surface Waters Groundwater
U.S. Environmental Protection Agency	US EPA	Office of Air and Radiation Office of Prevention and Toxic Substances Office of Pesticide Programs Office of Water

In general, biopesticides are naturally-occurring substances, often mixtures of biological substances obtained from natural sources, that have biological activities that are measurable, but are not pure substances with distinct chemical properties that are characteristic. Formulated active ingredients and products for commerce must be carefully tested and documented for mammalian toxicity (ingestion, pulmonary, dermal and eye irritation), genotoxicity, and ecotoxicty. For regulatory purposes, the term "biopesticides" usually includes (1) microbial pesticides - viral proteins, and their genetic material, bacteria, fungi, protozoa, and algae; (2) biochemicals, including food substances and food additives, pheromones, growth regulators, oils and numerous other substances found in

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch022

nature; and (3) plant incorporated protectants (PIPs). For this discussion, biopesticides are limited to biochemicals and extracts or partially purified substances of naturally-occurring materials. An additional requirement is that the registration submission must be submitted in its entirety.

By statutes within the Federal Food, Drug and Cosmetic Act (FFDCA) (*6*), Federal Insecticide , Fungicide and Rodenticide Act (FIFRA) (*7*), Food Quality Protection Act (FQPA) (*8*), and Pesticide Registration Improvement Act (PRIA) (*9*), the EPA has primary responsibility for registration of pest control and crop protection materials. Biopesticides are usually registered by the Biopesticides and Pollution Prevention Division (BPPD) of EPA. In comparison with registration of conventional chemical pesticides, biopesticide registrations for natural products are usually faster, less complex, and less costly when a considerable body of information already exists, and it is presented appropriately to the agency for use in the risk assessment process. Other biopesticides, such as microbials, and PIPs may have uncertainties which are not amenable to rapid evaluation and are not discussed here.

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch022

The Registration Process

Biochemical Classification

One should not assume that all natural products are biopesticides. Since most pesticides are conventional (synthetic) chemicals, and laws are written for registering such materials, BPPD employs a process which differentiates biopesticides from conventional ones. A Biopesticide Classification Committee reviews potential products and determines if BPPD will consider them for review. The primary points of consideration are:

1. Is the pesticidal material naturally-occurring?
2. Does it have a non-toxic mode of action on the pest to be controlled?
3. Is it less hazardous (toxic) than presently registered products?
4. Does it have activity that is harmful to non-target organisms?
5. Is it relatively non-persistent in the environment?

Overview

Because the registration of natural products as biopesticides differ markedly from conventional crop protection agents, procedures and processes have been modified in a manner which uses rational measurements that are more amenable to substances naturally occurring in the environment.

1. Request for consideration of material as a biopesticide.

   After preparing an informational summary describing pesticidal activity of the material, chemical properties or biological characteristics, a generalized description of the preparation, of the material describing its pesticidal activity, potential target species, chemical properties, and rationale for its consideration as a biopesticide, the registrant contacts BPPD Classification Committee and requests that the material be considered for classification as a biopesticide. After a brief discussion with the committee=s representative (process procedures, type of material to be considered, production, how measured, target organisms, etc.), a written request is submitted for classification.

2. After the classification as a biopesticide has been affirmed, the registrant may also request a pre-registration meeting to be held with the Biopesticides and Pollution Prevention Division review team (composed of management, regulatory and science reviewers) in order to clarify data requirements that may arise between the committee and/or the registrant. The pre-registration meeting guides the course of the registration process for a particular product. It generally focuses on what data are needed (mammalian toxicity B oral, pulmonary, dermal; genotoxicity; ecotoxicity; efficacy; etc.) for a quantitative risk assessment.

3. Other clarifications are usually confirmed (e.g., substances that are exempt from regulation and under what conditions). For example, cinnamon oil is an exempt product (*10*) while cinamaldehyde is not. Straight chain Lepidopteran pheromones are also exempt under specific conditions (*11*).

4. It is very important to have a well characterized product and protocol for its preparation. A brief listing of Guideline Studies for Biochemical Pesticides is listed in Table 2 (*12*).

   Although confirmation of research already conducted by others is often considered repeating known work, traceability to materials used in specific required studies may not be available. In other circumstances, some scientists may work with extracts as a whole entity rather than knowing what specific compound is the active ingredient. The active ingredient may be already registered; therefore, repeating the process through a natural source would probably not lead to a new product. Alternatively, an existing source of the biochemical may already exist that has specific chemical and physical characteristics or purity analysis specifications which may obviate the need

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch022

for growing, harvesting, extracting and fully characterizing it. Of course, there may be co-factors in the plant extract that strongly influence pesticidal activity. Therefore, it is important to have a well characterized product and protocol for its preparation.

Table 2. Guideline Studies for Biochemical Pesticides

40 CFR 40 158.690 Biochemical Pesticides Data Requirements
(a) Biochemical pesticide product analysis data requirements
(b) Biochemical pesticide residue data requirements
(c) Biochemical pesticide toxicology data requirements
(d) (Biochemical pesticide) Non-target organism fate and (environmental) expression data requirements

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch022

5. Waivers from the requirement to generate new data may sometimes be satisfied with data from credible published literature that may fulfill a study requirement when a rational explanation is provided for using the information in a quantitative risk assessment. Observational studies coupled with public literature and exposure information may be useful prior to the pre-registration meeting and EPA may be more likely to consider a waiver. If toxicology studies are required they will need to be conducted under Good Laboratory Practices (GLP) (*13*). The GLP studies are expensive since they require extensive documentation and record keeping. Once EPA has requested data from a GLP toxicology study, observational type studies are unlikely to be satisfactory and a waiver will not be successful.

6. Acute oral data requirements may sometimes be waived if the active ingredient is already a component of commonly consumed foods and the concentration of the active ingredient can be related to exposure. The assumptions are that the maximum number of applications at the maximum rate are all contained on the harvested portion of the crop without any washoff or degradation. By knowing the typical yields of a crop and the consumption data, a theoretical exposure can be calculated. This exposure can then be compared to the consumption of the active ingredient that may reside on the crop when it is consumed (see sample waiver justification below).

7. The Food and Drug Administration under 21 CFR Part 170. Food Additives designates chemicals Generally Regarded as Safe (GRAS). This GRAS designation may be part of a waiver request if justified by calculations of hazard and/or exposure based on the information in the appropriate literature. The GRAS citation, by itself, but cannot stand alone as a justification.

8. Dermal waivers may also be constructed by identifying the presence of the active ingredients in skin creams or foods. Discussions with a dermatologist or pharmacist may provide insights into potential problems associated with allergic reactions. A dermatologist may be able to assist with general knowledge of allergic reactions and specific information on hazards to avoid. Of particular interest is the sensitivity of women and children to substances not usually present in the immediate environment.

9. These data and information are integrated into a quantitative risk assessment for the determination of "reasonable certainty of no harm to vulnerable populations."

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch022

Justification for the waiver to fulfill a data requirement

The following is a sample waiver justification for an acute oral rat study. The degree of credence for justification varies from product to product, but they are listed here in order of the strongest to weakest justification. They are based on knowledge about the toxicity or relative amount of potential exposure. Data on structurally similar compounds reported in similar types of studies can also be cited. This information is used in the risk assessment analysis assures that the objective of "a reasonable certainty of no harm" is attained.

1. In the published literature, an acute oral rat study at five doses ranging from 0 to 5,000 mg of compound XYZ per kg rat determined that the LD_{50} of compound XYZ was 3,000 mg/kg .

2. In the published literature an acute oral toxicity study in the rat conducted at a single dose of 500 mg of compound XYZ was per kg rat determined that there were no adverse effects from compound XYZ at 500 mg/kg.

3. In the published literature an acute oral toxicity study in the rat at five doses ranging from 0 to 5,000 mg per kg rat determined that the LD_{50} of compound structurally related to compound $X^1Y^1Z^1$ was 3,000 mg/kg.

4. In the public literature an acute oral toxicity study in the rat conducted at a single dose of 500 mg per kg rat determined that there were no adverse effects from the compound structurally related to $X^1Y^1Z^1$.

5. Compound XYZ is naturally present in tree fruit and vegetables. By utilizing the maximum rate and maximum number of applications of XYZ,

and assuming that 100% of the applied product remains on the harvested portion of the crop, the maximum theoretical amount of compound XYZ is less than the concentration of compound XYZ is less than that naturally present within the crop. Based on USDA crop yield data and typical consumption data we have calculated the application of compound XYZ will contribute to 0.1% of that which is already consumed within the diet.

6. Compound XYZ is only applied at planting and has a half life of 3 days so compound XYZ is not likely to be present in the harvested crop.

7. Compound XYZ is only intended for use on hops, coffee and horseradish which represent crops with low dietary intake in children. Therefore the use of compound XYZ is not likely to impact the risk to this sensitive sub-population.

8. EPA has already approved compound XYZ as a list 4 inert product therefore this compound is already commonly used in pesticide formulations.

9. Compound XYZ is already approved by the Food and Drug Administration (21 CFR citation for XYZ) and it is classified as a Generally Regarded as Safe (GRAS) food additive. It is commonly found as a flavoring in soups and beverages.

10. Approximately 1 million pounds of compound XYZ is utilized per year as a direct food additive. By multiplying the known acreage of labeled crops and the maximum labeled rates of compound XYZ, the total potential to be applied would be 2,500 pounds.

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch022

Infrastructure and Potential Sources of Aid for Biopesticide Development

Biopesticides may become the preferred alternatives to some conventional pest control agents when used in home and/or commercial settings. In order to facilitate their development, university systems may assist in the pursuit of commercial interests through their Research and Development Office. The Office of Technology Transfer may assist with the patenting process and contacts with potential development partners. University systems vary in their freedom to pursue commercial interests of biopesticides.

While university scientists are familiar with obtaining grants for basic research, the transition from basic research to developing a marketable product is different. There are specific programs that can assist with efficacy, manufacturing and toxicology studies. The USDA IR-4 Project typically offers small grants to conduct efficacy research on biopesticides.

The EPA / OPP / BPPD / Pesticide Environmental Stewardship Program (PESP) has the goal of reducing risks associated with pesticide use in agricultural and non-agricultural settings in the United States and advocates adoption of biopesticides through a small grant program involving efficacy and promoting adoption of biopesticides by their cooperating partners.

The USDA has also developed a National Biological Control Laboratory in Stoneville, Mississippi with space for two pilot plants where scientists can cooperate with public and private organizations to test the practical applications of rearing techniques and foster commercial production, especially with small venture capital companies.

Other options may be developing a plan on licensing the rights of the product, setting up a campus-sponsored incubator company, or continuing technology transfer through federal Small Business Innovative Research (SBIR) grants.

The USDA invites science-based small business firms to submit research proposals under the SBIR Grant Program. The announcement can be found at http://www.fedgrants.gov/applicants/USDA-GRANTS:07010400/listing.html/. Through this program, USDA will support high-quality research or research and development proposals containing advanced concepts related to important scientific problems and opportunities that could lead to significant public benefit if the research is successful.

Objectives of the SBIR Program include stimulating technological innovation in the private sector, strengthening the role of small business in meeting Federal research and development needs, increasing private sector commercialization if derived from USDA-sponsored research and development efforts, and fostering and encouraging participation by women-owned and socially and economically disadvantaged small business firms in technological innovation. While not specified in their announcement, many small companies (including university spin-offs) have funded toxicology studies through this program.

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch022

The USDA also has a technology transition program known as the Cooperative Research and Development Agreement (CRADA). It is a written agreement between a private company and a government agency to work together on a project.

In 1986 and 1989, legislation was enacted as part of the Sevenson-Wydler Technology Innovation Act to enable federal laboratories to enter into Cooperative Research and Development Agreements (CRADAs) with private business and other entities. CRADAs provide the means to leverage research and development efforts and to create teams for solving technological and industrial problems. Through CRADAs, companies or groups of companies can work with one or more federal laboratories to pool resources and share risks in developing technologies.

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch022

Summary

Natural products as biopesticides provide challenges to the investigators, institutions, research sponsoring organizations, and agencies with responsibilities for evaluating research studies under codified guidelines and review procedures for conventional (synthetic) pesticides.

Although the characterization and registration of biopesticides may seem mysterious in comparison to the development and registration of conventional materials, a wide array of naturally-occurring substances have been brought to market. Many institutions, corporations and small businesses have brought natural products through the development and registration processes. Indeed, many such organizations believe that future maintenance of crop protection will continue with a greater emphasis with leads developed through greater understanding of natural products as crop protection agents.

References

1. Wei, Z.-M.; Laby, R.J.; Zumoff, C.H.; Bauer, D.W.; He, S.Y.; Collmer, A.; Beer, S.V. *Science* **1992,** *257*, **85-88.**
2. U. S. Environmental and Protection Agency, Biopesticides and Pollution Prevention Division. 2000. Harpin Protein (006477) Fact Sheet. http://www.epa.gov/biopesticides/biopesticides/ingredients/factsheets/factsheet_006477.htm.

3. Hofstein, R.; Chapple, A. In *Methods in Biotechnology*; Hall, F. R.; Menn, J. J., Eds.; Commercial Development of Biofungicides; Humana Press Inc.: Totowa, NJ, 1999; Vol. 5, pp 77- 102.
4. Bowers, R. C. In *Biological Control of Weeds with Plant Pathogens*; Charudattan, R.; Walker, H. L., Eds.; Wiley Interscience: Hoboken, NJ, 1982; pp 157-174.
5. Barton, D. H. R.; Meth-Cohn, O.; Nakanishi, K. *Comprehensive Natural Products Chemistry*; 9-Volume Set; Elsevier: St. Louis, MO, 1999; 8500 pp.
6. Federal Food, Drug and Cosmetic Act. Code of Federal Regulations. 1938 Title 21, Chapter 9.
7. Federal Insecticide, Fungicide and Rodenticide Act. 1947 Title 40 Code of Federal Regulations Chapter 1, Part 152.
8. Food Quality Protection Act. 1996. Public Law 104-170 104th Congress.
9. Pesticide Registration Improvement Act. Federal Insecticide, Fungicide and Rodenticide Act. Title 40 Code of Federal Regulations. Section 33 U.S.C. 7, 136w.8, 2004.
10. U. S. Environmental Protection Agency. Pesticide Registration (PR) Notice 2000-6. Notice to Manufacturers, Formulators, Producers and Registrants of Pesticide Products. Minimal Risk Pesticides Exempted under FIFRA Section 25(b) Clarification of Issues, 2000.
11. Exemptions for pesticides of a character not requiring FIFRA regulation. Code of Federal Regulations. 40 CFR Section 152.25 (b).
12. Biochemical Pesticide Test Guidelines. Code of Federal Regulations. 40 CFR Section 158.690.
13. Good Laboratory Practice Standards. Code of Federal Regulations. 40 CFR Part 160.

Downloaded by COLUMBIA UNIV on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ch022

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ap001

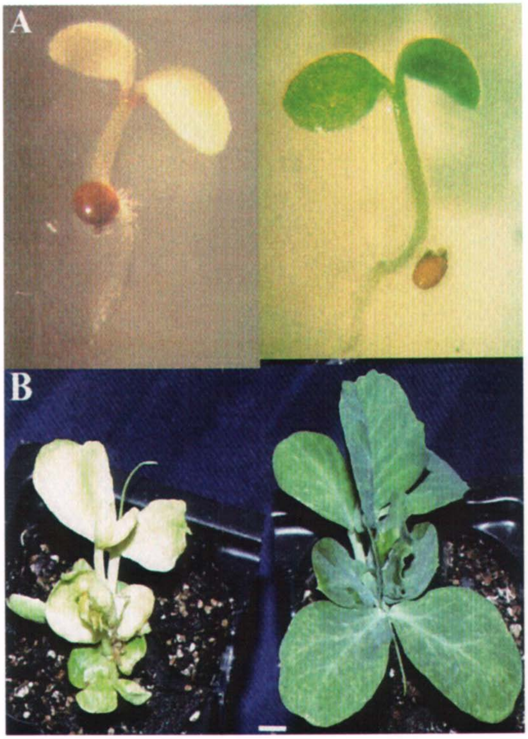


***Plate 19.3. Actinonin treatment of tobacco (A) and pea (B).
(A) Tobacco seeds (cv. Kentucky 14) were imbibed and seedlings cultured at room temperature with constant light (50 µmole.m-2.s-1) in Murashige and Skoog basal salts with 0.2% phytagel for 7 days in the absence (right) or presence (left) of 0.65 mM actinonin.
(B) Approximately 100-200 µl 6.5 mM actinonin was applied per pea plant in 0.1% Tween-20 (left). Control plants(right) were treated with 0.1% Tween-20. Means (n=12) of fresh (dry) weights were 0.72 g (0.11 g) and 0.43 g (0.063 g) for Tween-20-treated and actinonin-treated pea plants (16 day-old), respectively.***

In Natural Products for Pest Management; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2006.

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ap001

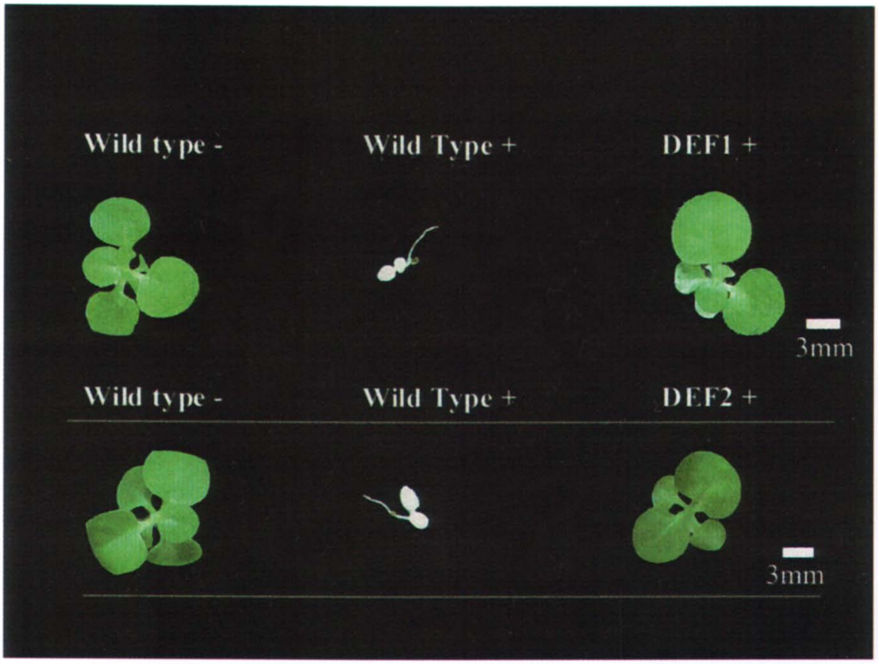


Plate 19.6. Actinonin treatment of Atdef over-expressing tobacco plants. Tobacco seeds of wild type orDEF1 or DEF2over-expressing plants were germinated in a nutrient- containing agarose medium with (+) or without (-) 1.2 mM actinonin. Photos were taken 42 days after germination.

Author Index

Baerson, Scott R., 255, 265
Bailey, Karen L., 37
Baron, J. J., 278
Boari, Angela, 76
Braverman, M. P., 278
Carr, Andy W., 37
Chauhan, Kamlesh R., 210
Clancy, Erin, 37
Coats, Joel, 168
Cook, Daniel, 265
Cutillo, Francesca, 24
D'Abrosca, Brigida, 24
Dayan, Franck E., 265
DellaGreca, Marina, 24
Derby, Jo-Ann, 37
Duke, Stephen O., 2, 152, 255, 265
Evidente, Antonio, 62
Fields, Paul G., 194
Fiorentino, Antonio, 24
Friebe, Annette, 233
Gemejieva, Nadejda G., 142
Gerwick, B. Clifford, 37
Gilbert, Jeffrey R., 37
Graupner, Paul R., 37
Hiradate, Syuntaro, 113
Holm, R. H., 278
Hou, Cai-Xia, 243
Jeschke, Peter, 128
Kagan, Isabelle A., 255
Kobaisy, Mozaina, 142
Komai, Koichiro, 182
Kunkel, D. L., 278
Lieb, Folker, 128
Mamonov, Leonid K., 142
Michel, Albrecht, 255
Morimoto, Masanori, 182
Mukanova, Gaukhar S., 142
Nanayakkara, N. P. Dhammika, 265
Pan, Zhiqiang, 265
Perry, Laura G., 99
Peterson, Chris, 168
Prause, Anja, 255
Raina, Ashok K., 210
Regiosa, Manuel J., 48
Rimando, Agnes M., 2, 265
Sánchez-Moreiras, Adela M., 48
Sassa, Takashi, 88
Sato, Daisuke, 88
Scheffler, Brian E., 255
Schrader, Kevin K., 142
Schultz, Gretchen, 168
Sekimoto, Hitoshi, 88
Siddall, Thomas L., 37
Sitpaeva, Gulnara T., 142
Takeuchi, Yasutomo, 88
Taylor, Wesley G., 194
Tellez, Maria R., 142
Velten, Robert, 128
Vivanco, Jorge M., 99
Vurro, Maurizio, 76
Wedge, David E., 142, 152
Weir, Tiffany L., 99
Westwood, James H., 220
Wiese, Welf-Burkhard, 128
Williams, Mark, 243
Yokota, Takao, 88
Yoneyama, Koichi, 88
Zarrelli, Armando, 24

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix001

Subject Index

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002

A

Abiotic stress
  effects of brassinosteroids on plant response, 237–239
  See also Brassinosteroids
Abscisic acid (ABA)
  possible allelochemicals, 122*t*
  structure, 123*f*
Acetolactate synthase (ALS), activity in plants, 83
Achillea millefolium
  phytotoxic activity, 148*t*
  plant in Kazakhstan, 144*t*
Aconitum leucostomum
  fungal growth inhibition on *Colletotrichum* species, 147, 149*t*
  plant in Kazakhstan, 144*t*
Acroptilon repens
  allelochemical identification from, 101–103
  production of allelochemicals, 102–103
  selectivity of allelochemicals for weed control, 108–109
  soil allelochemical concentrations, 106–107
  See also Allelochemistry; Knapweeds
Actinonin
  effects on plant peptide deformylase (DEF), 245–248
  engineered resistance to, by over-expression of *At*DEF genes, 248–250
  structure, 246*f*
  treatment of *At*DEF over-expressing tobacco plants, 251*f*
  treatment of tobacco and pea, 247*f*
  See also Peptide deformylase (DEF)
Active ingredients
  fungicidal, 138–139
  herbicidal, 136–138
  insecticidal, 133–135
  modern and complementary technologies, 139
  nematicidal, 135–136
  See also Modern crop protection
Activity
  concepts of specific and total, 114–116
  macrocidins, 42–43, 44*f*, 45*f*
  soil allelochemical, 106–107
Adaptation, plant mechanisms, 183
Aedes albopictus, natural product-based repellent, 7
Agrochemical industry, natural products, 129
Agropyron repens, inhibitory activity of possible allelochemicals as specific and total activity, 122*t*
Alantrypinone, alkaloid from *Aspergillus terreus*, 11
Alectrol, natural strigolactone, 90, 94*t*
Algicides
  activity of plant extracts from Kazakhstan, 145–146
  assay, 144
  natural products, 15–16
  See also Plants of Kazakhstan
Allelochemicals
  inhibitory activity of possible allelochemicals, 122*t*
  specific and total activities of reported, 121–122
  See also Sorgoleone
Allelochemistry

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002

- allelochemical identification from *Centaurea maculosa, C. diffusa,* and *Acroptilon repens*, 101–103
- allelochemical secretion from knapweeds, 104*f*
- *Arabinopsis* providing genetically tractable model for studying cellular effects of (-)-catechin, 106
- benefits of allelochemicals, 107–108
- (-)-catechin secretion from knapweeds, 103–104
- *C. maculosa, C. diffusa,* and *A. repens* producing different allelochemicals, 102–103
- ecological evidence of allelopathy in knapweeds, 102
- effects of allelochemicals on soil pathogens, 107–108
- identifying allelotoxins as natural herbicides, 100–101
- investigation of mode of action of (-)-catechin, 105–106
- plant derived compounds for pest management, 99–100
- precursors in flavonoid pathway, 103–104
- resistance mechanisms, 108–109
- selectivity of allelochemicals for weed control, 108–109
- soil allelochemical concentrations, 106–107
- structural modifications of (-)-catechin and activity, 105
- structure and activity of knapweed allelochemicals, 103–105

Allium cepa
- germination index (GI) of compounds from *Brassica fruticulosa*, 33, 34*f*
- GI of crude extract of *Cestrum parqui* on seeds of, 26*f*
- GI of cinnamic acid amides from *Chenopodium album* on seeds of, 28, 30*f*
- onion as standard target species, 25

Amblyomycin, fungicidal active ingredient, 138–139

Amino acids, parasitic weed management, 83–84

4-Aminoproline enantiomers
- phytotoxicity of *trans*-4-amino-D-proline, 66*t*
- phytotoxins from *Ascochyta caulina*, 63–65

Annonins, insecticidal active ingredients, 133–134

Anopheles gambiae
- identification of protein, 9
- repellent, 7

Anopheles mosquitoes, repellency of neem oil, 169

Anthraquinones, antifungal activity against *Colletotrichum* species, 160–161

9,10-Anthroquinone derivative, algicide, 16

Antialgal activity. *See* Plants of Kazakhstan

Antifeedant activity. *See* Field peas; Insect antifeedant activity

Antifungal activity
- biological evaluation, 155
- *See also* Fungicides; Plants of Kazakhstan

Apigenin, Formosan subterranean termite control, 9–10

Arabidopsis thaliana
- characterization of plant peptide deformylase genes (*At*DEF), 244–245
- investigation of mode of action of (-)-catechin, 105–106
- kinetic analysis of *At*DEF2 activity, 245, 246*f*
- microarrays, 256

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002

- model for studying cellular effects of (-)-catechin, 106
- *See also* Gene expression in *Arabidopsis thaliana*; Peptide deformylase (DEF)

Artemisia leucodes
- phytotoxic activity, 148*t*
- plant in Kazakhstan, 144*t*

Artemisia terrae-albae
- phytotoxic activity, 146, 148*t*
- plant in Kazakhstan, 144*t*

Ascaulitoxin
- phytotoxicity by leaf puncture assay, 66*t*
- phytotoxin from *Ascochyta caulina*, 63–65

Ascochyta caulina
- ascaulitoxin, 64
- biological characterization of phytotoxins, 65
- description, 63
- greenhouse and field experiments, 65–67
- isolation and chemical characterization of phytotoxins, 63–65
- phytotoxicity of ascaulitoxin and *trans*-4-amino-D-proline by leaf puncture assay, 66*t*
- phytotoxin analytical methods and application as biomarkers, 67
- stereochemistry of ascaulitoxin and 4-aminoproline enantiomers, 64*f*
- structures of phytotoxins, 64*f*
- *See also* Phytotoxins

Ascochyta sonchi
- biological characterization of ascosonchine, 72–73
- description, 71
- effect of ascosonchine in leaf puncture assay, 73*t*
- isolation and chemical characterization of phytotoxins, 71–72
- structure of ascosonchine, 72*f*
- *See also* Phytotoxins

Ascosonchine
- biological characterization, 72–73
- effect in leaf puncture assay, 73*t*
- isolation and characterization, 71–72
- structure, 72*f*
- *See also Ascochyta sonchi*; Phytotoxins

Aspergillus terreus, alkaloids from, 11

Aucubin, algicidal activity, 146

Avermectins, insecticidal compounds, 10–11

Azadirachta indica
- compounds with insecticidal properties, 9
- repellency of mosquitoes, 169

Azadirachtin
- insect antifeedant, 184
- structure, 10
- triterpenoid as repellent, 9

Azoxystrobin
- detached leaf assay, 158
- fungicide, 13

B

Bacillis thuriengensis, transgenes encoding toxins, 16

Bayer CropScience
- amblyomycin, 138–139
- annonins, 133–134
- cripowellins, 134
- enniatins, 138
- fungicidal β-methoxyacrylates, 130–131
- herbicidal active ingredients, 136–138
- herbicidal phosphinic acids, 131–132
- insecticidal active ingredients, 133–135
- insecticidal pyrethroids, 130

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002

- nematicidal active ingredients, 135–136
- omphalotin A, 135–136
- products with natural origin, 130–132
- pyrenocines, 136–137
- rocaglamide, 134–135
- search for new sources of natural products, 132–139
- taccalonolides, 136
- thiolactomycin, 137–138
- *See also* Modern crop protection

7,8-Benzoflavone
- resistance of *Gaillardia aristata*, 109
- secretion from knapweeds, 104*f*
- testing effects on plant growth, 109

Benzoic acid, herbicide, 6

Benzopyranones, insect antifeedant activity, 190, 191*f*

Benzoquinones
- antifungal activity against *Colletotrichum* species, 160–161
- *Cyperus* species, 187–188

2-Benzoxazolinone (BOA)
- activities of antioxidant enzymes superoxide dismutase (SOD) and peroxidase (POD), 58–59
- ATPase activity, 49–50
- carbon:nitrogen (C:N) ratio, 52, 53*f*
- experimental designs, 49–50
- free proline and osmolality in treated lettuce plants, 55*f*
- free proline content, 56, 57*f*
- height and root length of treated lettuce plants, 50*f*
- leaf biomass, leaf necromass, and total leaf proteins in treated lettuce plants, 50, 51*f*
- lettuce seeds, 49
- malondialdehyde content (MDA), 58–59
- measurement of SOD and POD activities, 49
- osmoregulation and proline concentration, 54–55
- photosynthesis and transpiration measurements, 49
- photosynthetic process, 57
- plant metabolism, 52–54
- polyamines contents, 59
- primary and secondary effects, 48–49
- proline accumulation, 55, 56*f*
- putrescine catabolism, 56, 57*f*
- relative water content (RWC), water potential, and osmolality in treated lettuce plants, 53–54
- root biomass, root length, and total root proteins in treated lettuce plants, 52*f*
- root morphology, 51
- RWC, leaf biomass, and transpiration rate in treated lettuce plants, 54, 54*f*
- specificity for single BOA treatment, 51–52
- specific leaf area (SLA), 54, 55*f*
- spermine, spermidine, and putrescine contents, 59
- stomatal density (SD), 54, 55*f*
- water use efficiency, 56–57

Bialaphos
- bioactivation, 5
- herbicide, 131–132
- specific activity, 115

Bilanafos, herbicide, 131–132

Bioactive compounds
- concepts of specific and total activity, 114–116
- *See also* Activity; Isolation strategies

Bioassays
- C8 extracts by elution of C8 silica column with methanol, 195, 196*f*, 197
- choice-test arena, 178–179
- choice test using vetiver oil, 212, 213*f*

  - feeding and mortality, using vetiver oil, 213, 214*f*, 216*t*, 217*t*
  - flour disk bioassay, 195, 197
  - German cockroach and house fly, 171–172
  - mosquito repellency, 172–174
  - repellency methods, 171–174
  - tunneling assay using vetiver oil, 212–213, 214, 216*t*
- Bioautography
  - optimum performance laminar chromatography (OPLC) and, of *Pimpinella* spp., 162
  - screening system, 155–156
  - *See also* Direct bioautography; Fungicides
- Biochanin A, Formosan subterranean termite control, 10
- Biological agents, weed control, 63
- Biology. *See* Molecular biology
- Biomarkers
  - application of *Ascochyta caulina* phytotoxins, 67
  - toxic metabolites by weed pathogens as, 82–83
- Biopesticide development, infrastructure and potential sources of aid, 285–287
- Biopesticides
  - monoterpenoids, 210
  - natural products as, 279–281
  - term, 280–281
  - *See also* Vetiver oil
- Biopesticides and Pollution Prevention Division (BPPD)
  - registration, 281
  - registration process, 281–285
- *Blattella germanica*
  - German cockroach and house fly bioassay, 171–172
  - German cockroach and house fly repellency, 174, 175*t*
  - *See also* Insect repellents
- Bleaching herbicides
  - natural products, 38
  - *See also Phoma macrostoma*
- *Botrytis cinerea*
  - 96-well microbioassay, 157
  - activity of osthol, 14
  - activity of surangin B, 14
- *Brassica fruticulosa*
  - comparison of bioactive lignans with pendimethalin, 33, 35*f*
  - description, 29
  - germination index (GI) of compounds from, on lettuce, tomato, and onion, 33, 34*f*
  - isolation and identification of lignans and neolignans, 29, 31–33
  - metabolites inhibiting germination and growth, 24–25
  - structures of dilignan and sesquilignans from, 32*f*
  - structures of lignans, 31*f*
  - structures of neolignans, 31*f*
- *Brassica oleracea*, inhibitory activity of possible allelochemicals as specific and total activity, 122*t*
- Brassinosteroids
  - activation of mitogen-activated protein kinase (MAPK) in rice, 240
  - analysis of *Lychnis viscaria* extract, 235
  - biological activity by sensitive rice lamina inclination test, 235–236
  - effects on plant response to abiotic stress, 237–239
  - experiments for studying resistance-inducing effects of, 236–237
  - future prospects, 240
  - induction of disease resistance in plants by, 234–237
  - isolation procedure for, containing fraction of *L. viscaria* seed extract, 236*f*
  - molecular aspects of, signal transduction, 239–240

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002

  peroxidase activity stimulation for cucumber, 235
  structures of 24-epi-secasterone and 24-epi-castasterone, 237*f*
  studies of, effects on plant response to abiotic stress, 238*t*
Broomrapes
  control difficulties, 77
  germination, 89
  parasitic weed species, 76, 221–222
  See also Orobanche species; Parasitic plant management

C

Callicarpa americana, terpenoids from, 7, 8
Callicarpenal, terpenoid as mosquito repellent, 7, 8
Callistemon spp., leptospermone, 5, 6
Camphor, natural pesticide, 12
Cancer chemotherapy, vinca alkaloids, 153–154
Cantharidin, herbicide analogue, 6
Carbon:nitrogen ratio, 2-benzoxazolinone (BOA) treated lettuce, 52–53
(-)-Catechin
  effects of, and (+)-catechin on soil pathogens, 107–108
  investigation of mode of action of, 105–106
  model for studying cellular effects, 106
  phytotoxicity of (+)- and (-)-isomers, 103–104
  plant flavanoid, 103
  secretion from knapweeds, 104*f*
  structural modifications and activity, 105
  testing effects on plant growth, 109
  See also Allelochemistry
Catnip
  contact repellency of northern house mosquito, 177*t*
  mosquito repellency, 179
  Nepeta cataria as insect repellent, 170
  percentage repellency of northern house mosquito, 176*f*, 177*f*
  percent repellency in choice-test arena bioassay, 175*t*
  residual repellency of mosquito, 178
  See also Insect repellents
CAY-1 fungicidal steroidal saponin, isolation and identification, 161, 162*f*
Centaurea diffusa
  allelochemical identification from, 101–103
  ecological evidence of allelopathy in knapweeds, 102
  effects of allelochemicals on soil pathogens, 107–108
  investigation of mode of action of (-)-catechin, 105–106
  production of allelochemicals, 102–103
  soil allelochemical concentrations, 106–107
  structure and activity of allelochemicals, 103–105
  See also Allelochemistry; Knapweeds
Centaurea maculosa
  allelochemical identification from, 101–103
  ecological evidence of allelopathy in knapweeds, 102
  effects of allelochemicals on soil pathogens, 107–108
  investigation of mode of action of (-)-catechin, 105–106
  production of allelochemicals, 102–103
  soil allelochemical concentrations, 106–107

- structure and activity of allelochemicals, 103–105
- *See also* Allelochemistry; Knapweeds

Ceratitis capitata, penitrems A-D and F against, 11

Cercospora kikuchii, rhamnolipids against, 13

Cestrum parqui
- description, 25
- germination index (GI), 25
- GI of C_{13} nor-isoprenoids from, on lettuce seeds, 27*f*
- GI of crude extract of, on lettuce, tomato and onion, 26*f*
- isolation and structure elucidation of C_{13} nor-isoprenoids, 25–27
- metabolites inhibiting germination and growth, 24–25
- structures of C_{13} nor-isoprenoids from, 26*f*

Chenopodium album
- description, 27
- germination index (GI) of crude extract of, on seeds of lettuce, tomato and onion, 28*f*
- GI of cinnamic acid amides from, on seeds of lettuce, tomato and onion, 28, 30*f*
- isolation and structure elucidation of cinnamic acid amide, 28, 29*f*
- metabolites inhibiting germination and growth, 24–25
- structures of cinnamic acid amides, 29*f*

Choice test bioassay
- *Coptotermes formosanus* workers, 212, 213*f*
- vetiver oil, 214, 215*t*

Chrysanthemum cinerariaefolium, pyrethroids, 183

Chrysanthemum species, insecticides, 8

1,4-Cineole, herbicide, 6

Cinerin, *Chrysanthemum* species, 8

Cinmethylin, herbicide, 6

Cinnamic acid amide
- germination index (GI) of, from *Chenopodium album* on lettuce, tomato, and onion seeds, 30*f*
- isolation and structure elucidation, 28, 29*f*
- structures, 29*f*
- *See also Chenopodium album*

1-*O*-*cis*-Cinnamoyl-β-D-glucopyranose (*cis*-CG)
- plant growth inhibiting compounds in *Spiraea thunbergii*, 124–125
- possible allelochemical, 122*t*
- structure, 123*f*

Cladosporium cucumerinum, rhamnolipids against, 13

Clematis species, plants in Kazakhstan, 144*t*

Cnaphalocrocis medinalis, diet of treated rice leaves, 9

Cockroaches
- nepetalactone isomers, 11, 12
- repellency, 174, 175*t*
- repellent bioassay, 171–172
- *See also* Insect repellents

Colletotrichum gloesporioides, activity of osthol, 14

Colletotrichum musae, activity of osthol, 14

Colletotrichum orbiculare, rhamnolipids against, 13

Colletotrichum species
- 96-well microbioassay, 157
- fungal growth inhibition of active plants on, 147, 149*t*
- fungicidal assays, 145
- naturally occurring quinones, 160–161

Combinatorial chemistry, high throughput screens, 3–4

Concentrations, soil allelochemical, 106–107

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002

Cooperative Research and Development Agreement (CRADA), 287
Coptotermes formosanus
  biopesticides, 211
  choice test bioassay of, workers, 212, 213*f*
  feeding and mortality assay of, workers, 213, 214*f*
  means to control, 9–10
  tunneling assay of, workers, 212–213
  See also Vetiver oil
Coriolus versicolor, medicarpin against, 15
Cotton, transgenes for insect control, 16–17
Cotylenins, false germination stimulants, 93–95
Cripowellins, insecticidal active ingredients, 134
Crop protection
  activation of plant defense mechanisms, 234
  See also Modern crop protection
Crop resistance
  developing parasite-resistant crops, 222–223
  host-parasite connection, 223–224
  See also Orobanche species
Cucumber, induction of disease resistance by brassinosteroids, 234–237
Culex nigripalpus, natural product-based repellent, 7
Culex pipiens
  insects for repellency bioassay, 172
  See also Mosquito repellents
Culex pipiens pallens, activity of α-terpinene against, 7
Cyanamide
  possible allelochemical, 122*t*
  structure, 123*f*
Cyclohexadepsipeptides (CHDPs), *N*-methylated, enniatins, 138
Cylindrocarpon destructans, rhamnolipids against, 13
Cyperaquinone, antifeedant from *Cyperus* spp., 185, 186*f*
Cypermethrin, commercial insecticide, 183
Cyperus spp.
  cyperaquinone, 185, 186*f*
  dihydrocyperaquinone, 185, 186*f*
  hydroxydietrichequinone, 187–188
  insect antifeedants in, 185–188
  remirol, 185, 186*f*
  scabequinone, 185, 186*f*
  simple benzoquinone from, 187–188
  structure activity relationships of dihydrobenzofurans, 185–187
  See also Insect antifeedant activity
Cytochalasins
  biological characterization of, by *Pyrenophora semeniperda*, 68–69
  effect on root elongation of tomato and wheat seedlings, 70*f*
  Phoma exigua var. *heteromorpha*, 69–70
  toxins as herbicides, 78
  See also Phytotoxins

D

Deacetylgedunin, targeting gut enzymes, 9
Deacetylnimbin, targeting gut enzymes, 9
DEET (*N,N*-diethyl-*m*-toluamide)
  contact repellency of northern house mosquito, 177*t*
  insect repellent, 7
  mosquito exposure to DEET and elemol, 179–180
  mosquito repellent, 169, 179–180
  percentage repellency of northern house mosquito, 176*f*, 177*f*

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002

percent repellency in choice-test arena bioassay, 175*t*
residual repellency of mosquito, 178
See also Insect repellents
Defense chemicals, acting against insects, 183
Dehydrofusaric acid, production and seed germination inhibition, 82–83
cis-Dehydromatricaria ester (*cis*-DME)
from *Solidago altissima*, 124
possible allelochemical, 122*t*
structure, 123*f*
Dehydrosoyasaponin I (D-I), isolation, 204–206
Delphinium semibarbatum
fungal growth inhibition on *Colletotrichum* species, 147, 149*t*
plant in Kazakhstan, 144*t*
Detached leaf assay
fungicide evaluations, 162, 164
fungicide evaluations *in planta*, 158
See also Fungicides
4,15-Diacetoxyscirpenol, germination inhibitor, 95, 97*f*
4,15-Diacetylnivalenol, germination inhibitor, 95, 97*f*
Dicamba, herbicide, 6
N,N-Diethyl-*m*-toluamide (DEET)
contact repellency of northern house mosquito, 177*t*
insect repellent, 7
mosquito exposure to DEET and elemol, 179–180
mosquito repellent, 169, 179–180
percentage repellency of northern house mosquito, 176*f*, 177*f*
percent repellency in bioassay test, 175*t*
residual repellency of mosquito, 178
See also Insect repellents
Dihydrobenzofurans, structure activity relationship (SAR), 185–187
Dihydrocyperaquinone, antifeedant from *Cyperus* spp., 185, 186*f*
2,4-Dihydroxy-1,4-benzoxazin-3-one (DIBOA)
possible allelochemical, 122*t*
structure, 123*f*
2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA)
possible allelochemical, 122*t*
structure, 123*f*
L-3,4-Dihydroxyphenylalanine (L–DOPA)
possible allelochemical, 122*t*
structure, 123*f*
Dilignan
structure, 32*f*
See also Brassica fruticulosa
Direct bioautography
antifungal compounds from *Ruta graveolens*, 161
matrix bioautography showing location of active antifungal zones, 159*f*
screening system, 155–156
strawberry anthracnose resistance, 160
See also Fungicides
Disease resistance, induction in plants by brassinosteroids, 234–237
Durantanins
Duranta repens, 122*t*
structures, 123*f*

E

Elemol
contact repellency of northern house mosquito, 177*t*
major component of essential oil, 171
mosquito exposure to DEET and, 179–180

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002

natural pesticide, 12
  percentage repellency of northern house mosquito, 176*f*, 177*f*
  residual repellency of mosquito, 178
  See also Insect repellents
Endothall, herbicide, 6
Enniatins, fungicidal active ingredient, 138
Environmental stress, effects of brassinosteroids on plant response, 237–239
Eremurus fuscus
  fungal growth inhibition on *Colletotrichum* species, 147, 149*t*
  plant in Kazakhstan, 144*t*
Eremurus inderiensis
  fungal growth inhibition on *Colletotrichum* species, 147, 149*t*
  phytotoxic activity, 148*t*
  plant in Kazakhstan, 144*t*
Eucalyptus citriodora, PMD (p-menthane-3,8-diol), 7
Eucalyptus globulus, essential oil as repellent, 7
Euphorbia latifolia, plant in Kazakhstan, 144*t*
Experimental design, 2-benzoxazolinone (BOA), 49–50
Expressed sequence tag (EST) study. *See* Sorgoleone
Extraction, natural product preparation, 129

F

Feeding and mortality assay
  Coptotermes formosanus workers, 213, 214*f*
  vetiver oil, 214, 216*t*, 217*t*
Fermentation, natural product preparation, 129
Ferula leiophylla, fungal growth inhibition on *Colletotrichum* species, 147, 149*t*
Field experiments, *Ascochyta caulina* phytotoxins, 65–67
Field peas
  activity in fractions by elution of C8 silica column, 195
  additional fractionation of C8-2a, 204
  antifeedant and insecticidal activity of pea soyasaponins and synergistic mixtures, 207*t*
  C8 extracts in methanol, 196*f*
  chemical structures of minor components of fraction C8-2a, 203*f*
  dose-response experiment of C8 fractions, 197, 198*f*
  experimental C8 extracts and insect bioassays, 195, 197
  flash chromatography with silica cartridge, 199*t*
  flour from, insecticidal to stored-product insects, 195
  fractionation of C8 powder, 197, 199
  high performance liquid chromatography (HPLC) confirming soyasaponin I, 200*f*, 201*f*
  HPLC and mass spectral data for components of active fraction C8-2a, 202*t*
  identification of components, 199, 202, 204
  isolation of dehydrosoyasaponin I (D-I), 204–206
  prominent daughter ions during collision-induced dissociation experiments, 205*t*
  soyasaponin I (S–I) as major component, 199, 202
  synergy tests, 206, 207*t*
Flavonoid pathway, precursors and phytotoxicity, 103–104

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002

Flavonoids
  insect antifeedant activity of benzopyranones, 190, 191*f*
  polymethylated tree, from *Gnaphalium* sp., 188–190
  rotenoid, 184
Formosan subterranean termite (FST)
  biopesticides, 211
  means to control, 9–10
  See also Vetiver oil
Frenching disease, tobacco, 83
Fungal metabolites
  false germination stimulants, 93–95
  germination inhibitors, 95, 97*f*
Fungicidal active ingredients
  activity of plant extracts against *Colletotrichum* species, 147, 149*t*
  amblyomycin, 138–139
  assays, 145
  enniatins, 138
  See also Active ingredients; Plants of Kazakhstan
Fungicides
  96-well microbioassay, 157–158
  anthracnose resistance in strawberry, 160
  antifungal compounds from *Ruta graveolens*, 161
  application of microbioassay to *Phytophthora nicotianae*, 161–162
  azoxystrobin, 158, 164*t*
  biography coupled with optimum performance laminar chromatography (OPLC), 156–157
  biological evaluation of antifungal activity, 155
  CAY-1 fungicidal steroidal saponin, 161, 162*f*
  compounds acting directly on pathogens, 153–154
  compounds inducing resistance mechanisms in plants, 154
  densimetric analysis of nandrolone products by thin layer chromatography (TLC) and OPLC, 163*f*
  detached leaf assay for evaluations *in planta*, 158
  detached leaf assays, 162, 164
  direct bioautography, 155–156, 159–164
  2D-TLC fingerprint, 159–160
  inducing systemic acquired resistance (SAR), 154
  materials and methods, 155–158
  matrix bioautography, 159*f*
  β-methoxyacrylates, 130–131
  microbioassay evaluation, 161–162
  naturally occurring quinones, 160–161
  natural products, 13–15
  OPLC and bioautography of *Pimpinella* spp., 162
  OPLC resolution and bioautography with *Colletotrichum* species, 163*f*
  pathogen production, 155
  sampangine, 162*f*, 164*t*
Fusaric acid, production and seed germination inhibition, 82–83
Fusarium graminearum, activity of osthol, 14
Fusarium oxysporum
  96-well microbioassay, 157
  activity of osthol, 14
  activity of surangin B, 14
  medicarpin and maackiain, 15
  mycoherbicides, 84
Fusicoccins, false germination stimulants, 93–95

G

Gaillardia aristata, resistance mechanism, 109
Gedunin, targeting gut enzymes, 9

Gene expression in *Arabidopsis thaliana*
  benzoxazolin-2(3H)-one (BOA), 260*f*
  genes from three recognized phases of detoxification responding to BOA treatments, 262
  materials and methods, 261
  microarray hybridization, real time PCR, and analysis, 261
  plant material, growth conditions, and treatments, 261
  response to BOA, 260–263
  RNA isolation, 261
  validation of microarray data, 263
Gene expression in *Saccharomyces cerevisiae*
  expression patterns, 259–260
  fold change in expression of some ergosterol biosynthesis genes, 260*t*
  materials and methods, 257–259
  microarray analysis, 259
  outline of ergosterol biosynthetic pathway, 258*f*
  response to sterol biosynthesis inhibitors (SBIs), 257
  RNA isolation and microarray hybridization, 258–259
  yeast strains, media, and growth condition, 257–258
Genomics approach. *See* Sorgoleone
Geranium collinum
  fungal growth inhibition on *Colletotrichum* species, 147, 149*t*
  phytotoxic activity, 146, 148*t*
  plant in Kazakhstan, 144*t*
Germination inhibitors
  parasite seed germination, 95, 97*f*
  toxins from fungal pathogens, 78–81
Germination stimulants
  active jasmonate-type, 96*f*
  chemical nature, 85
  chemical structures of natural strigolactones, 90*f*
  cotylenins and fusicoccins, 93–95
  effect of nutrients in growth media on production, 93
  false, 93–95
  fungal metabolites, 93–95
  general structures of fusicoccins, 96*f*
  jasmonates, 93–95
  multiple reaction monitoring (MRM) chromatograms of soybean root exudates, 91, 92*f*
  natural, 90–93
  Orobanche minor by root exudates, 91*f*
  parasitic plants, 84–85
  production by various host plants, 94*t*
  spontaneous germination, 89
  strigolactones, 90–93
Glufosinate
  natural product as herbicide, 4, 131–132
  structure, 5
Glycosyl juglone
  possible allelochemical, 122*t*
  structure, 123*f*
Gnaphalium affine, isolation of insect antifeedants from, 188–190
Governmental scientists, proposal, research and publication, 278–279
Greenhouse experiments, *Ascochyta caulina* phytotoxins, 65–67

H

Haplophyllum sieversii
  fungal growth inhibition on *Colletotrichum* species, 147, 149*t*
  phytotoxic activity, 146, 148*t*
  plant in Kazakhstan, 144*t*
Harpagoside, algicidal activity, 146

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002

Haustorium
  host-parasite connection, 221
  See also Orobanche species
Health and Human Services, pesticide oversight, 280*t*
Hederagenin, molluscicide, 15, 16
Hedge apple tree. *See* Osage orange
Herbicidal active ingredients
  pyrenocines, 136–137
  thiolactomycin, 137–138
  See also Active ingredients
Herbicides
  analogues of natural products, 5, 6
  identifying allelotoxins as natural, 100–101
  natural products, 4–6, 77–82
  phosphinic acids, 131–132
  secondary metabolites as, 24–25
  See also Allelochemistry; Bleaching herbicides; *Phoma macrostoma*
Hesperetin, fungicidal activity, 14–15
High throughput screens
  combinatorial chemistry, 3–4
  ultra high, for new natural products, 139
Host-parasite connection
  haustorium, 221
  physiology, 223–224
  See also Orobanche species
House flies
  nepetalactone isomers, 11
  repellency, 174, 175*t*
  repellent bioassay, 171–172
  See also Insect repellents
Hydantocidin, herbicide, 6
6-*O*-(4'-Hydroxy-2'-methylene-butyroyl)-1-*O*-*cis*-cinnamoyl-β-D-glucopyranose (*cis*-BCG)
  plant growth inhibiting compounds in *Spiraea thunbergii*, 124–125
  possible allelochemical, 122*t*
  structure, 123*f*
17-Hydroxyazadiradione, targeting gut enzymes, 9
Hydroxydietrichequinone, *Cyperus* species, 187–188
Hydroxyphenyl pyruvate dioxygenase (HPPD)
  inhibition by bleaching herbicides, 38
  role in biosynthetic pathway of tocopherols and plastoquinone from tyrosine, 43
  triketones inhibiting, 5
8-Hydroxyquinoline
  effects on soil pathogens, 107–108
  secretion from knapweeds, 104*f*
  testing effects on plant growth, 109

I

Imidacloprid, commercial insecticide, 183
In-house strain collection, identifying new natural products, 139
Insect antifeedant activity
  antifeedants in *Cyperus* spp., 185–188
  azadirachtin, 184
  benzopyranones, 190, 191*f*
  correlation between, and dihydrobenzofuran derivatives, 187*f*
  hydroxydietrichequinone from *Cyperus javanicus*, 187–188
  insecticidal phytochemicals and commercial insecticides, 183*f*
  isolation of, from *Gnaphalium affine*, 188–190
  natural products with insecticidal activity, 184*f*
  nicotine, 183
  physostigmine, 183
  pyrethroids, 183
  QSAR of benzopyranone for, 190, 191*f*
  rotenone, 184

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002

scanning electron microscopy (SEM) of woolly filaments on *Gnaphalium* leaf surface, 189*f*
simple benzoquinone from *Cyperus* spp., 187–188
spinosad, 184
structure activity relationship (SAR) of dihydrobenzofurans, 185–187
woolly surface of *G. affine* leaf, 189*f*

Insecticidal active ingredients
  annonins, 133–134
  cripowellins, 134
  rocaglamide, 134–135
  See also Active ingredients

Insecticides
  commercial, 183
  natural products, 7–12
  pyrethroids, 130

Insect repellency, mechanism, 9

Insect repellents
  catnip, 170, 175*t*
  contact irritancy, 171
  contact repellency of northern house mosquito, 177*t*
  Culex pipiens colony for repellency testing, 172
  N,N-diethyl-*m*-toluamide (DEET), 7, 169, 175*t*
  elemol, 171
  German cockroach and house fly bioassay, 171–172
  German cockroach and house fly repellency, 174, 175*t*
  mosquito repellency, 172–174, 176, 177*f*
  mosquito residual repellency bioassay, 173–174
  nepetalactone isomers in catnip, 170*f*, 175*t*
  Osage orange, 170–171
  percentage and contact repellency bioassay, 172–173
  percent repellency, 172
  percent repellency in choice-test arena bioassay, 175*t*
  repellency bioassay methods, 171–174
  residual repellency, 178, 180

Intermedeol, terpenoid as mosquito repellent, 7, 8

Iris ruthenica, plant in Kazakhstan, 144*t*

Iris songorica
  fungal growth inhibition on *Colletotrichum* species, 147, 149*t*
  plant in Kazakhstan, 144*t*

Isolation strategies
  following specific activity as indicator, 116–118
  following total activity as indicator, 118–119, 120*f*

J

Jasmolin, *Chrysanthemum* species, 8

Jasmonates
  false germination stimulants, 93–95
  most active jasmonate-type germination stimulants, 96*f*

Juglans nigra
  inhibitory activity of possible allelochemicals as specific and total activity, 122*t*
  plant growth inhibition under, 121

Juglone
  possible allelochemical , 122*t*
  structure, 123*f*

Juniperus pseudosabina
  fungal growth inhibition on *Colletotrichum* species, 147, 149*t*
  plant in Kazakhstan, 144*t*

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002

K

Kazakhstan. *See* Plants of Kazakhstan
Knapweeds
  7,8-benzoflavone secretion, 104*f*
  (-)-catechin secretion, 104*f*
  ecological evidence of allelopathy in, 102
  8-hydroxyquinoline secretion, 104*f*
  precursors in flavonoid pathway, 103–104
  production of allelochemicals, 102–103
  secretion of allelochemicals, 104*f*
  structure and activity of, allelochemicals, 103–105
  See also Allelochemistry

L

Lactuca sativa
  germination index (GI) of compounds from *Brassica fruticulosa*, 33, 34*f*
  GI of crude extract of *Cestrum parqui* on seeds of, 26*f*
  GI of C_{13} nor-isoprenoids from *C. parqui* on seeds, 27*f*
  GI of cinnamic acid amides from *Chenopodium album* on seeds of, 28, 30*f*
  lettuce as standard target species, 25
  See also Cestrum parqui
Lavandula officinalis, essential oil as repellent, 7
Leaf biomass, necromass and total proteins, 2-benzoxazolinone (BOA) treated lettuce, 50–51
Leptospermone, analogue of natural product, 5, 6
Leptospermum scoparium, leptospermone, 5, 6
Lettuce. *See* 2-Benzoxazolinone (BOA); *Lactuca sativa*
Lignans
  comparison of bioactive lignans from *Brassica fruticulosa* with pendimethalin, 33, 35*f*
  isolation and identification in *B. fruticulosa*, 29, 31–33
  structures from *B. fruticulosa*, 31*f*
  See also Brassica fruticulosa
Ligupurpuroside A, algicidal activity, 146
Limonoids from neem tree, targeting gut enzymes, 9
Linaria vulgaris, plant in Kazakhstan, 144*t*
Lychnis viscaria
  analysis of resistance-inducing seed extract, 235
  24-epi-secasterone and 24-epi-castasterone, 237*f*
  isolation procedure for brassinosteroid, 236*f*
  resistance inducing effects, 234
  See also Brassinosteroids
Lycopersicon esculentum
  germination index (GI) of compounds from *Brassica fruticulosa*, 33, 34*f*
  GI of crude extract of *Cestrum parqui* on seeds of, 26*f*
  GI of cinnamic acid amides from *Chenopodium album* on seeds of, 28, 30*f*
  tomato as standard target species, 25

M

Maackiain, fungicidal activity, 15
Maclura pomifera. *See* Osage orange
Macrocidins
  activity, 42–43, 44*f*, 45*f*
  bleaching natural products, 38

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002

  hydroxylated derivatives, 42
  mode of action, 43, 46
  reversal experiments using homogentisic acid, 44*f*, 45*f*
  structure, 38*f*
  structures of naturally occurring derivatives, 40*f*
  substituted, using ring-closing olefin metathesis (RCM), 41
  synthesis of, and unnatural analogs, 40–42
  See also Phoma macrostoma
Macrophoma kawatsukai, activity of osthol, 14
Magnaporthe grisea, rhamnolipids against, 13
Maize, transgenes for insect control, 16–17
Malondialdehyde (MDA) content, 2-benzoxazolinone (BOA) treated lettuce, 58–59
Mammea longifolia, surangin B, 14
Manzamine alkaloids, insecticidal compounds, 11, 12
Marine organisms, insecticidal compounds, 11, 12
Mechanisms
  activation of plant defense mechanisms, 234
  adaptation for plants, 183
  insect repellency, 9
  resistance and tolerance, to natural herbicides, 108–109
Medicarpin, fungicidal activity, 14–15
p-Menthane-3,8-diol (PMD)
  endorsement, 7, 12
  mosquito repellent, 7
Methionine aminopeptidase (MAP), protein processing, 244
β-Methoxyacrylates, fungicides, 130–131
Microarrays
  gene expression, 263
  identifying candidates for mode of action in pesticide, 256
  See also Gene expression in *Arabidopsis thaliana*; Gene expression in *Saccharomyces cerevisiae*
Microbioassay
  96-well, 157–158
  evaluation, 161–162
  See also Fungicides
Mode of action
  investigation of, of (-)-catechin, 105–106
  macrocidins, 43, 46
  model for 2-benzoxazolinone (BOA), 48–49
  spinosyns, 10
Modern crop protection
  amblyomycin, 138–139
  annonins, 133–134
  cripowellins, 134
  differentiation of natural products (NPs) in "The Pesticide Manual", 129*f*
  enniatins, 138
  fungicidal active ingredients, 138–139
  fungicidal β-methoxyacrylates, 130–131
  herbicidal active ingredients, 136–138
  herbicidal phosphinic acids, 131–132
  influence of NPs in design of synthetic active ingredients, 132
  in-house strain collection, 139
  insecticidal active ingredients, 133–135
  insecticidal pyrethroids, 130
  modern and complementary technologies, 139
  nematicidal active ingredients, 135–136
  omphalotin A, 135–136
  preparation methods for NPs, 129
  products from Bayer CropScience with natural origin, 130–132

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002

- pyrenocines, 136–137
- requirements for use of NPs, 129
- rocaglamide, 134–135
- search for new sources of NPs, 132–139
- taccalonolides, 136
- thiolactomycin, 137–138
- ultra high throughput screening (UHTS) bioassays, 139

Molecular biology
- natural products for pest management, 4
- pest management, 16–17

Momilactone B
- *Oryza sativa*, 122*t*
- structure, 123*f*

Mosquito repellents
- common chemicals, 169
- compounds from genus *Piper*, 8
- contact repellency of northern house mosquito, 177*t*
- DEET (*N,N*-diethyl-*m*-toluamide), 7, 169
- exposure to DEET and elemol, 179–180
- insects, 172
- investigations with catnip and osage orange, 179
- mosquito residual repellency bioassay, 173–174
- percentage and contact repellency bioassay, 172–173
- percentage repellency, 173
- repellency of catnip, osage orange essential oil, elemol and DEET, 174, 176*f*, 177*f*
- residual repellency, 178, 180
- terpenoids from *Callicarpa americana*, 7, 8
- *See also* Insecticides; Insect repellents

Mucuna pruriens, inhibitory activity of possible allelochemicals as specific and total activity, 122*t*

Musca domestica
- German cockroach and house fly bioassay, 171–172
- house fly repellency, 174
- nepetalactone isomers, 11, 12
- percent repellency of repellents in choice-test arena bioassay, 175*t*
- *See also* Insect repellents

T-Muurolol
- Formosan subterranean termite control, 9
- structure, 10

Mycoherbicides, obtaining virulent, due to toxin overexpression, 84

N

Naphthoquinones, antifungal activity against *Colletotrichum* species, 160–161

Naringenin, fungicidal activity, 14–15

National Biological Control Laboratory, USDA, 286

Natural products
- agrochemical industry, 129
- algicides, 15–16
- biopesticides, 279–281
- fungicides, 13–15
- herbicides, 4–6, 77–82
- insecticides and insect repellents, 7–12
- molluscicides, 15
- nematocides, 16
- pest management, 16–17
- preparation, 129
- rodenticides, 15
- search for new sources, 132–139
- sources of molecules for pesticides, 3
- *See also* Active ingredients; Fungicides; Insect antifeedant activity; Insect repellents; Modern crop protection; Parasitic plant management

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002

Neem limonoids, targeting gut enzymes, 9
Neem tree
  compounds with insecticidal properties, 9
  repellency of neem oil for mosquitoes, 169
Nematicidal active ingredients
  omphalotin A, 135–136
  taccolonolides, 136
  See also Active ingredients
Nematocides, natural products, 16
Neolignans
  isolation and identification in *Brassica fruticulosa*, 29, 31–33
  structures from *B. fruticulosa*, 31*f*
  See also Brassica fruticulosa
Nepeta cataria
  catnip as insect repellent, 170
  See also Insect repellent
Nepetalactone isomers
  catnip as insect repellent, 170
  German cockroach and house fly repellency, 179
  insect control, 11, 12
  percentage repellency of northern house mosquito, 176*f*, 177*f*
  percent repellency in choice-test arena bioassay, 175*t*
  See also Insect repellents
Nepeta pannonica
  phytotoxic activity, 146, 148*t*
  plant in Kazakhstan, 144*t*
cis-Nerol, Formosan subterranean termite control, 10
Nicotiana rustica, nicotine, 183
Nicotiana tobacum, inhibition of peptide deformylase by actinonin, 248
Nonoic acid, natural compound as herbicide, 5
Nootkatone
  Formosan subterranean termite control, 9
  sesquiterpene in vetiver oil, 211*f*
  structure, 10

O

Ochlerotatus triseriatus, natural product-based repellent, 7
Oil of lemon eucalyptus, insecticide, 7
O-methyltransferases. *See* Sorgoleone
Omphalotin A, nematicidal active ingredient, 135–136
Oncopeltus fasciatus, penitrems A-D and F against, 11
Onion. *See Allium cepa*
Operation Full Stop, termite control, 9–10
Optimum performance laminar chromatography (OPLC)
  natural product isolation, 156–157
  See also Fungicides
Orobanche minor, germination stimulation on, by root exudates, 91*f*
Orobanche species
  advantages and risks of sarcotoxin IA, 229
  control difficulties, 77
  current control strategies, 222–223
  development of parasite-resistance crops, 222–223
  germination, 89
  impact of, on economically important crops, 221–222
  natural germination stimulants, 90–93
  natural products for control of, 224–230
  O. aegytpiaca growing on tobacco, 223*f*
  physiology of host-parasite connection, 223–224
  possible mechanisms of action of sarcotoxin IA against, 228–229

- potential for sarcotoxin IA for crop improvement, 225–226
- potential mycoherbicides from *O. ramosa* plants, 79–80
- sarcotoxin, 225–229
- scopoletin in sunflower parasite-resistant crop varieties, 224
- tobacco expressing sarcotoxin IA showing enhanced resistance to, 226–228
- worst parasitic weed, 76
- *See also* Parasitic plant management

Orobanchol, natural strigolactone, 90, 94*t*

Oryza sativa, inhibitory activity of possible allelochemicals as specific and total activity, 122*t*

Osage orange
- contact repellency of northern house mosquito, 177*t*
- insect repellency of essential oil, 170–171
- mosquito repellency, 179
- percentage repellency of northern house mosquito, 176*f*, 177*f*
- *See also* Insect repellents

Oscillatoria perornata, algicidal assay, 144

Osmolality, 2-benzoxazolinone (BOA) treated lettuce, 53–54

Osmoregulation, 2-benzoxazolinone (BOA) treated lettuce, 54–55

Osthol, fungicide, 14

P

Parasitic plant management
- amino acids, 83–84
- chemical nature of germination stimulants, 85
- competing with crops for resources, 221
- deoxynivalenol, 78–79
- inhibition of germination, 78–81
- introduction of toxins for broomrape, 81–82
- macrocyclic trichothecenes, 81*f*
- natural compounds as herbicides, 77–82
- neosolaniol monoacetate, 81*f*
- *Orobanche ramosa* plants, 79–80
- phytotoxins from fungal pathogens of crops, 78
- problem, 76–77
- seed germination stimulants, 84–85
- T-2 toxin, 78–79
- toxic metabolites as biomarkers, 82–83
- trichothecenes, 78, 79*f*
- verrucarins, 80, 81*f*
- virulent mycoherbicides due to toxin overexpression, 84
- *See also Orobanche* species

Patents, natural phytotoxins, 6

Pea. *See* Field peas; Peptide deformylase (DEF)

Pelargonic acid, natural compound as herbicide, 5

Pendimethalin, comparison of bioactive lignans from *Brassica fruticulosa* with, 33, 35*f*

Penicillium crustosum, insecticidal activity of mycotoxins, 11

Peptide deformylase (DEF)
- actinonin treatment of *At*DEF over-expressing tobacco plants, 251*f*
- actinonin treatment of tobacco and pea, 247*f*
- *Arabidopsis thaliana* DEFs (*At*DEFs), 244–245
- effects of actinonin on, 245–248
- effects of actinonin on D1 protein synthesis in tobacco seedlings, 249*f*
- engineered resistance to actinonin, 248–250
- functional consequences of, inhibition, 248

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002

  inhibition of, by actinonin in tobacco, 248
  initial characterization of plant, 244–245
  kinetic analysis of *At*DEF2 activity, 245, 246*f*
  protein processing, 244
  structure of actinonin, 246*f*
  Western analysis of leaf extracts from tobacco with engineered over-expression of *At*DEF, 250*f*
Periplaneta Americana, nepetalactone isomers, 11, 12
Peroxidase (POD), activity in 2-benzoxazolinone (BOA) treated lettuce, 49, 58–59
Persistence, soil allelochemical, 106–107
Pesticide discovery
  natural products as sources, 3
  obstacles to natural product-based, 3
  pharmaceutical industry, 2–3
Pesticide Environmental Stewardship Program (PESP), 286
Pest management
  molecular biology and natural products, 16–17
  natural products, 4, 169
  plant derived compounds, 99–101
  See also Allelochemistry
Pharmaceutical industry
  natural products discovery efforts, 4
  pesticide discovery, 2–3
Phoma macrostoma
  activity, 42–43, 44*f*, 45*f*
  amphidicolin, 39
  bioassay directed isolation, 39
  bleaching activity in extracts, 38
  description, 39
  hydroxylated macrocidin derivatives, 42
  isolation from diseased thistle plants, 38–39
  macrocidins, 38
  mode of action of macrocidins, 43, 46
  phomalirazine, 39
  structures of amphidicolin and phomalirazine, 39*f*
  structures of naturally occurring macrocidin derivatives, 39, 40*f*
  substituted macrocidins using ring-closing olefin metathesis (RCM), 41
  synthesis of macrocidins and unnatural analogs, 40–42
Phoma species
  new cytochalasins from *P. exigua* var. *heteromorpha*, 69–70
  structures of phytotoxins, 69*f*
  See also Phytotoxins
Phomopsis obscurans, 96-well microbioassay, 157
Phomopsis viticola, 96-well microbioassay, 157
Phosphinic acids, herbicides, 131–132
Phosphinothricin
  natural product as herbicide, 4
  specific activity, 115
  structure, 5
Photosynthesis, 2-benzoxazolinone (BOA) effects on lettuce, 49, 57
Physiology, host-parasite connection, 223–224
Physostigma venenosum, physostigmine, 183
Phytoalexins, plant defense compounds, 14–15
Phytochemicals, insecticidal, 183
Phytohormones. *See* Brassinosteroids
Phytophthora capsici
  activity of osthol, 14
  rhamnolipids against, 13
Phytophthora cryptogea, rhamnolipids against, 13
Phytophthora nicotianae, application of microbioassay, 161–162

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002

Phytophthora spp., microbioassay, 157–158
Phytotoxic activity
  assays, 144–145
  extract of plants from Kazakhstan, 146–147, 148*t*
  See also Plants of Kazakhstan
Phytotoxins
  Ascochyta caulina, 63–67
  Ascochyta sonchi, 71–73
  definition, 63
  fungal pathogens of crops, 78
  identifying allelotoxins as natural herbicides, 100–101
  Pyrenophora and *Phoma*, 67–71
  See also Allelochemistry
Picaridin, insecticide, 7
Piper genus, mosquito repellent compounds from, 8
Pipernonaline, mosquito repellent from genus *Piper*, 8
Pipgulzarine, mosquito repellent from genus *Piper*, 8
Piptigrine, mosquito repellent from genus *Piper*, 8
Pipzorine, mosquito repellent from genus *Piper*, 8
Plant-derived compounds. *See* Allelochemistry
Plant growth inhibition
  black walnut canopy (*Juglans nigra*), 121
  compounds in *Spiraea thunbergii*, 124–125
Plant metabolism, 2-benzoxazolinone (BOA) treated lettuce, 52–54
Plants
  chemicals for defense and communication, 153
  compounds inducing resistance mechanisms in, 154
  mechanisms of adaptation, 183
  See also Fungicides
Plants of Kazakhstan
  algicidal activity, 145–146
  algicidal assays, 144
  bioassays, 144–145
  fungicidal activity, 147, 149*t*
  fungicidal assays, 145
  materials and methods, 143–145
  phytotoxicity activity, 146–147, 148*t*
  phytotoxicity assays, 144–145
  plant collection, 143, 144*t*
  preparation of extracts, 143
Plasmopara lactucae-radicis, rhamnolipids against, 13
Plastoquinone
  analogue of natural product, 5, 6
  role of hydroxyphenyl pyruvate dioxygenase (HPPD) in synthesis, 43
PMD (p-menthane-3,8-diol)
  endorsement, 7, 12
  mosquito repellent, 7
Proline concentration, 2-benzoxazolinone (BOA) treated lettuce, 54–55, 56*f*
Protein-rich pea flour
  activity, 195
  See also Field peas
Pseudomonas aeruginosa, rhamnolipids, 13
Pyrenocines, herbicidal active ingredients, 136–137
Pyrenophora species
  biological characterization of cytochalasins by *P. semeniperda*, 68–69
  effect of cytochalasins on root elongation of tomato and wheat seedlings, 70*f*
  isolation and characterization of *P. semeniperda* phytotoxins, 68
  phytotoxins, 67–68
  P. semeniperda, 68
  structures of phytotoxins, 69*f*
  See also Phytotoxins
Pyrethins, *Chrysanthemum* species, 8
Pyrethroids, insecticidal product, 130

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002

Pythium aphanidermatum, rhamnolipids against, 13

Q

Quercetin, defense compound, 189
Quinones, antifungal activity against *Colletotrichum* species, 160–161

R

Registration process
  biochemical classification, 281
  guideline studies for biochemical pesticides, 283*t*
  justification for waiver to fulfill data requirement, 284–285
  overview, 281–284
Regulatory considerations
  Biopesticides and Pollution Prevention Division (BPPD), 281
  Cooperative Research and Development Agreement (CRADA), 287
  department and agencies with oversight affecting pesticides, 280*t*
  Health and Human Services, 280*t*
  infrastructure and potential sources of aid for biopesticide development, 285–287
  National Biological Control Laboratory, 286
  natural products as biopesticides, 279–281
  Office of Technology Transfer, 285
  Pesticide Environmental Stewardship Program (PESP), 286
  registration process, 279, 281–285
  Small Business Innovative Research (SBIR), 286
  university and governmental research, 278–279
  U.S. Department of Agriculture, 280*t*
  U.S. Department of Commerce, 280*t*
  U.S. Department of the Interior, 280*t*
  U.S. Environmental Protection Agency (EPA), 279, 280*t*
  See also Registration process
Relative water content (RWC), 2-benzoxazolinone (BOA) treated lettuce, 53–54
Remirol, antifeedant from *Cyperus* spp., 185, 186*f*
Repellency bioassay methods
  German cockroach and house fly bioassay, 171–172
  mosquito repellency, 172–174
  See also Insect repellents
Resistance, potential mechanism of, to natural herbicides, 108–109
Resistance mechanisms, compounds inducing, in plants, 154
Rhamnolipids, broad-spectrum fungicides, 13
Rhizoctonia solani
  activity of osthol, 14
  activity of surangin B, 14
  rhamnolipids against, 13
Rice leaffolder, diet of treated rice leaves, 9
Rocaglamide, insecticidal active ingredients, 134–135
Root morphology, 2-benzoxazolinone (BOA) treated lettuce, 51–52
Rosa canina, inhibitory activity of possible allelochemicals as specific and total activity, 122*t*
Rosmarinus officinalis, essential oil as repellent, 7
Rotenoid, flavonoids, 184
Rotenone, natural pesticide, 12

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002

Ruta graveolens, antifungal compounds from, 161

S

Saccharomyces cerevisiae
  microarrays, 256
  See also Gene expression in *Saccharomyces cerevisiae*
Saccharopolyspora spinosa, spinosyns, 10, 11
Salannin, targeting gut enzymes, 9
Sampangine
  detached leaf assay, 162, 164*t*
  structure, 162*f*
  See also Fungicides
Sarcotoxin IA
  advantages and risks, 229
  possible mechanisms of action against *Orobanche*, 228–229
  potential use for crop improvement, 225–226
  tobacco expressing, for enhanced *Orobanche* resistance, 226–228
  See also Orobanche species
Scabequinone, antifeedant from *Cyperus* spp., 185, 186*f*
Scilliroside, rodenticide, 15, 16
Sclerotinia sclerotiorum, activity of osthol, 14
Scopoletin, sunflower resistance to *Orobanche cernua*, 224
Screening systems
  direct bioautography, 155–156
  optimum performance laminar chromatography (OPLC), 156–157
  ultra high throughput screening (UHTS) bioassay, 139
Secondary metabolites, useful natural herbicides, 24–25
Selectivity, allelochemicals for weed control, 108–109
Selenastrum capricornutum, algicidal assay, 144
Serantrypinone, alkaloid from *Aspergillus terreus*, 11
Sesquilignans
  structures, 32*f*
  See also Brassica fruticulosa
Sesquiterpenes, vetiver oil, 211*f*
Signal transduction
  molecular aspects of brassinosteroid, 239–240
  See also Brassinosteroids
Small Business Innovative Research (SBIR), objectives, 286
Soil pathogens, benefits of allelochemicals, 107–108
Solidago altissima
  cis-dehydromatricaria ester (*cis*-DME) from, 124
  inhibitory activity of possible allelochemicals as specific and total activity, 122*t*
  structure of *cis*-DME, 123*f*
Sorghum species. *See* Sorgoleone
Sorgolactone, natural strigolactone, 90, 94*t*
Sorgoleone
  allelochemical by several *Sorghum* species, 266
  analogue of natural product, 5, 6
  candidate sequences in *S. bicolor* root hair EST database, 272*t*
  cDNA library construction and expressed sequence tag (EST) data analysis, 267
  chemical structure, 266*f*
  clone G10_84, 274–275
  current understanding of biosynthetic pathway, 269, 270*f*
  experimental system choice of *S. bicolor* cultivar (BTX623), 271
  expressed sequence tag (EST) analysis, 269
  generation of EST database, 271

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002

  heterologous expression of recombinant *O*-methyltransferases, 268–269
  location of biosynthetic enzymes, 269
  Magic Gene Discovery software and BLAST searches, 271–272
  materials and methods, 267–269
  O-methyltransferase enzyme assays, 269
  O-methyltransferase substrate specificity, 272, 273*f*
  5-pentadecatrienyl resorcinol, 274–275
  proposed biosynthetic pathway, 270*f*
  real-time polymerase chain reaction (RT-PCR), 267–268
  root hair cell isolation, 271*f*
  top 10 BLAST results for clone G10_84, 274*t*
Soyasaponin I (S-I)
  antifeedant and insecticidal activity, 206, 207*t*
  high performance liquid chromatography (HPLC), 200*f*, 201*f*
  identification, 199, 202, 204
  See also Field peas
Spathulenol, terpenoid as mosquito repellent, 7, 8
Specific activity
  concept, 114–115
  inhibitory activity of possible allelochemicals as, 122*t*
  isolation procedure following, as indicator, 116–118
  phosphinothricylalanylalanine (bialaphos), 115
  reported allelochemicals, 121–122, 123*f*
  See also Total activity
Specific leaf area (SLA), 2-benzoxazolinone (BOA) treated lettuce, 54, 55*f*
Spinosad, insect neurotoxin, 184
Spinosyns
  activity against insect pests, 10
  structures, 11
Spiraea thunbergii
  inhibitory activity of possible allelochemicals as specific and total activity, 122*t*
  plant growth inhibiting compounds in, 124–125
Stilbenes, growth regulatory effect, 11–12
Stomatal density (SD), 2-benzoxazolinone (BOA) treated lettuce, 54, 55*f*
Strategies. *See* Isolation strategies
Strawberry, anthracnose resistance, 160
Streptomyces avermitilis, avermectins, 10–11
Streptomyces spp.
  bialaphos, 5
  phosphinothricin, 4
  specific activity, 115
Stress in plants. *See* Brassinosteroids
Striga hermonthica, T-2 and deoxynivalenol against, 79
Striga species
  control strategies, 222
  germination, 89
  natural germination stimulants, 90–93
  seed germination, 93–95
Strigol, natural strigolactone, 90, 94*t*
Strigolactones
  chemical structures, 90*f*
  natural germination stimulants, 90–93
  production by various host plants, 94*t*
Strobilurin fungicides
  derivatives of β-methoxyacrylates, 130–131
  synthetic analogs, 13
Strobilurus tenacellus, fungicides, 13

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002

Structure activity relationships (SAR)
  dihydrobenzofurans, 185–187
  macrocidins, 42–43, 44*f*, 45*f*
  QSAR of benzopyranone for insect antifeedant activity, 190, 191*f*
Strychnine, rodenticide, 15, 16
Suicidal germination, strategy, 89
Sulcotrione, analogue of natural product, 5, 6
Sunflower, scopoletin in, parasite-resistant crops, 224
Superoxide dismutase (SOD), activity in 2-benzoxazolinone (BOA) treated lettuce, 49, 58–59
Surangin B, fungicide, 14
Synergy tests, pea soyasaponins, 206, 207*t*
Systemic acquired resistance, plant protection, 154

T

Taccolonolides, nematicidal active ingredients, 136
Tamarix arceuthoides
  phytotoxic activity, 146, 148*t*
  plant in Kazakhstan, 144*t*
Termites, means to control, 9–10
Terpenoids
  Callicarpa americana, 7, 8
  insect repellents and feeding deterrents, 210
  synthesis in potato for insect control, 17
α-Terpinene, repellent activity, 7
3,3',5,5'-Tetrahydroxy-4-methoxystilbene, growth regulatory effect, 11–12
Thin layer chromatography (TLC), bioautography screening system, 155–156
Thiolactomycin, herbicidal active ingredient, 137–138
Thymus vulgaris, essential oil as repellent, 7
Tobacco
  Frenching disease, 83
  induction of disease resistance by brassinosteroids, 234–237
  inhibition of peptide deformylase by actinonin, 248
  Orobanche aegyptiaca growing on, 223*f*
  See also Peptide deformylase (DEF)
Tocopherols, role of hydroxyphenyl pyruvate dioxygenase (HPPD) in synthesis, 43
Tolerance, potential mechanism of, to natural herbicides, 108–109
Tomato. *See Lycopersicon esculentum*
Total activity
  concept, 115–116
  definition, 116
  inhibitory activity of possible allelochemicals as, 122*t*
  isolation procedure following, as indicator, 118–119, 120*f*
  reported allelochemicals, 121–122, 123*f*
  See also Specific activity
Toxin overexpression, obtaining virulent mycoherbicides, 84
Toxins. *See* Phytotoxins
Transgenes, insect control, 16–17
Transpiration, 2-benzoxazolinone (BOA) effects on lettuce, 49, 54
Trichothecenes
  chemical structure of macrocyclic, 81*f*
  deoxynivalenol, 78–79
  T-2 toxin, 78–79
Tunneling assay
  Coptotermes formosanus workers, 212–213
  vetiver oil, 214, 216*t*
Tyrosine, role of hydroxyphenyl pyruvate dioxygenase (HPPD) in

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002

- synthesis of tocopherols and plastoquinone from, 43

U

Ultra high throughput screening (UHTS) bioassays, new natural products, 139
University scientists, proposal, research and publication, 278–279
U.S. Department of Agriculture, pesticide oversight, 280*t*
U.S. Department of Commerce, pesticide oversight, 280*t*
U.S. Department of the Interior, pesticide oversight, 280*t*
U.S. Environmental Protection Agency (EPA)
- pest control and crop protection, 281
- pesticide oversight responsibilities, 280*t*
- preparing information for review, 279
- *See also* Regulatory considerations

Usnic acid, analogue of natural product, 5, 6

V

Verbascoside, algicidal activity, 146
Verbascum blattaria, plant in Kazakhstan, 144*t*
Verbascum songaricum
- fungal growth inhibition on *Colletotrichum* species, 147, 149*t*
- plant in Kazakhstan, 144*t*

Vetiver oil
- bioactivity of modified, to Formosan subterranean termites, 214
- choice test bioassay, 212, 213*f*
- consumption by *Coptotermes formosanus* workers of treated filter paper, 216*t*
- distribution of *C. formosanus* workers on treated vs. control filter paper disks in choice test, 215*t*
- feeding and mortality, 213, 214*f*
- materials, 212
- mortality of *C. formosanus* workers by exposure to/feeding on treated filter paper, 217*t*
- repellency to Formosan subterranean termites, 217
- sesquiterpenes in, 211*f*
- steam distillation of roots from *Vetiveria zinzanoides*, 211
- tunneling and time to tunnel by *C. formosanus* workers through treated sand, 216*t*
- tunneling assay, 212–213
- vetivols or sesquiterpenols, 211

Vicia faba, vicine, 14
Vicia villosa, inhibitory activity of possible allelochemicals as specific and total activity, 122*t*
Vicine, natural fungicides, 14
Vinca alkaloids, cancer chemotherapy, 153–154
Vulgarone B, molluscicide, 15, 16

W

Water potential, 2-benzoxazolinone (BOA) treated lettuce, 53–54
Water use efficiency, 2-benzoxazolinone (BOA) treated lettuce, 56–57
Weed control
- identifying allelotoxins as natural herbicides, 100–101
- selectivity of allelochemicals, 108–109
- soil allelochemical concentrations, 106–107

Weeds
  biological agents, 63
  control, 62–63
  phytotoxins, 63
  See also Phytotoxins
Witchweeds (*Striga* spp.), germination, 89

X

Xylylcarb, commercial insecticide, 183

Downloaded by 89.163.34.136 on July 6, 2012 | http://pubs.acs.org
Publication Date: May 25, 2006 | doi: 10.1021/bk-2006-0927.ix002